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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/811,492	03/19/2004	Shannon Marshall	34098/US/2	1818

7590 09/06/2006

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EXAMINER	
XIE, XIAOZHEN	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 09/06/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/811,492	MARSHALL, SHANNON	
	Examiner	Art Unit	
	Xiaozhen Xie	1646	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *20 July 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-11* is/are pending in the application.

4a) Of the above claim(s) *5,7,10 and 11* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,6,8 and 9* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *02 December 2004* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

Applicant's amendments of the specification and the drawings filed 2 December 2004 have been entered. Applicant's amendment of the claims filed 20 July 2006 is acknowledged.

Election/Restrictions

Applicant's election of Group I, claims 1-9, without traverse, and species election of IFNb, in the response received 20 July 2006 is acknowledged.

Claims 1-11 are pending. Claims 5, 7, 10 and 11 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Claims 1-4, 6, 8 and 9 are under examination to the extent they read on the elected species.

Specification

The disclosure is objected to because of the following informalities:

The paragraph [045] is missing a number after USSN_____.

Correction is required.

Claim Objections

Claim 3 is objected to because of the following informalities: claim 3 recites non-elected inventions. Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-4, 6, 8 and 9 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The claims are directed to a composition comprising a prodrug agent comprising: a) a protein that induces unwanted side effects due to undesired activity at or close to the site of administration; b) a substantially non-immunogenic polymer; c) a covalent labile linker between said protein and said polymer. The claims are broad in that the recitation of "a protein" encompasses a genus of molecules, known or unknown, with a diverse range of structures and functions. What applicant has described in the specification are polymer-conjugated therapeutic proteins, such as thrombopoietin (TPO), BMP-7, IFN-β and ciliary neorotrophic factor (CNTF). Applicant has not described the genus of polymer-conjugated proteins that can be used as prodrug. There is no teaching regarding the relationship of structure to function, such as what structure feature these molecules have. Further, there is no requirement that these molecules have any particular function. Thus, the claims encompass a genus of molecules, which vary substantially in composition, and could have very different structural and functional characteristics from the conjugation products that Applicant has disclosed.

To provide adequate written description and evidence of possession of a claimed genus, the specification must provide sufficient distinguishing identifying characteristics of the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making of the claimed product, or any combination thereof. In this case, there is not even identification of any particular portion of the structure that must be conserved. Accordingly, in the absence of sufficient recitation of distinguishing identifying characteristics, the specification does not provide adequate written description of the claimed genus.

Vas-Cath Inc. v. Mahurkar, 19USPQ2d 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the 'written description' inquiry, *whatever is now claimed*." (See page 1117.) The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (*See Vas-Cath* at page 1116). As discussed above, the skilled artisan cannot envision the detailed chemical structure of the encompassed genus of peptides, and therefore, conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the method of isolation. Adequate written description requires more than a mere statement that is part of the invention and reference to a method of isolating it. The compound itself is required. See *Fiers v. Revel*, 25 USPQ2d 1601 at 1606 (CAFC 1993) and *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016.

One cannot describe what one has not conceived. See *Fiddes v. Baird*, 30 USPQ2d 1481 at 1483. In *Fiddes*, claims directed to mammalian FGF's were found to be unpatentable due to lack of written description for that broad class. The specification provided only the bovine sequence.

Therefore, only polymer-conjugated therapeutic proteins including TPO, BMP-7, IFN-β and CNTF, but not the full scope of the claimed conjugation products, is adequately described in the disclosure.

Claims 1-4, 6, 8 and 9 are further rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a prodrug agent comprising a PEG-conjugated IFN-β, does not reasonably provide enablement for a prodrug comprising any polymer-conjugated protein. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

The claims are broad in that they encompass a prodrug comprising any protein conjugated to a polymer through a covalent labile linker. The specification defines "protein" as a molecule comprising at least two covalently attaches amino acids, which includes proteins, polypeptides, oligopeptides and peptides [020]. Thus, the recitation of "a protein" encompasses a genus of molecules, known or unknown, with a diverse range of structures and functions. The specification discloses a PEG-conjugation IFNβ. The specification, however, does not provide any guidance for making or using other prodrugs comprising any protein conjugated to a polymer through a covalent labile linker. There is no teaching regarding the relationship of structure to function, such as

what structure feature these molecules have. Further, there is no requirement that these molecules have any particular function, such as what disease theses molecules can be employed in treating. While the prior art (El Tayar et al., WO 99/55377) describes the same molecules, it fails to provide compensatory guidance. Further, not all of the therapeutic proteins can be chemically modified to achieve the benefits such as enhanced plasma half-life, reduced toxicity, and increased drug stability and solubility. For example, Kozlowski et al. (Biodrugs, 2001, 15(7):419-429) state that "unfortunately, insufficient knowledge of protein structure or the inability to selectively place the PEG moiety may hinder attempts to eliminate protein immunogenicity (pp. 420, right column, 1st paragraph). Since the claims encompass a large genus of molecules with no requirement for structure and activity, and the specification does not define what these molecules will be, one of skill in the art would evaluate all non-exemplified polymer-conjugated proteins for therapeutic uses. Thus, undue experimentation would be required for the artisan to make and use the invention as broadly claimed.

Due to the large quantity of experimentation necessary to generate the nearly infinite number of polymer-conjugated proteins recited in the claims and screen same for therapeutic uses, the lack of direction/guidance presented in the specification regarding which structural features are required in order to provide therapeutic uses, the absence of working examples directed to same, the complex nature of the invention, the state of the prior art which establishes the unpredictability of the effects of protein structure on function, and the breadth of the claims which fails to recite any structural

and functional limitations, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1 and 8 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The claims recite "a substantially non-immunogenic polymer" or ""said polymer substantially interferes with the activity of said protein". The phrase "substantially" is held to be indefinite because the specification lacks some standard for measuring the degree intended.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-4, 6, 8 and 9 rejected under 35 U.S.C. 102(b) as being anticipated by El Tayar et al. (WO 99/55377, publication date 4 November 1999). WO 99/55377 teaches PEG-IFN-β conjugates, where a PEG moiety is covalently bound to Cys^{17} of human

IFN-β. WO 99/55377 teaches a pharmaceutical composition comprising the PEG-IFN-β conjugates for treating infections, tumor and autoimmune and inflammatory diseases (pp. 11 line 32 to pp. 12, line 8). WO 99/55377 teaches that IFN-β has antiviral activity and can also stimulate natural killer cells against neoplastic cells (pp. 1, lines 17-19). WO 99/55377 also teaches that IFN-β and the PEG-IFN-β conjugates are capable of inducing cellular proliferation or differentiation (see Example 3). Therefore, WO 99/55377 anticipates the instant claims.

Conclusion

NO CLAIM IS ALLOWED.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Xiaozhen Xie, Ph.D whose telephone number is 571-272-5569. The examiner can normally be reached on M-F, 8:30-5.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary B. Nickol, Ph.D. can be reached on 571-272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Xiaozhen Xie, Ph. D.
August 30, 2006

GARY B. NICKOL, PH.D.
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Notice of References Cited	Application/Control No. 10/811,492	Applicant(s)/Patent Under Reexamination MARSHALL, SHANNON	
	Examiner Xiaozhen Xie	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO 99/55377 A	11-1999	would	El Tayar et al.	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Kozlowski et al., Biodrugs, 2001, 15(7):419-429.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

LEADING ARTICLE

BioDrugs 2001; 15 (7): 419-429
1173-8804/01/0007-0419/$22.00/0

Development of Pegylated Interferons for the Treatment of Chronic Hepatitis C

Antoni Kozlowski, Stephen A. Charles and *J. Milton Harris*

Shearwater Corporation, Huntsville, Alabama, USA

Abstract

The chemical attachment of poly(ethylene glycol) [PEG] to therapeutic proteins produces several benefits, including enhanced plasma half-life, lower toxicity, and increased drug stability and solubility. In certain instances, pegylation of a protein can increase its therapeutic efficacy by reducing the ability of the immune system to detect and mount an attack on the compound.

A PEG-protein conjugate is formed by first activating the PEG moiety so that it will react with, and couple to, the protein. PEG moieties vary considerably in molecular weight and conformation, with the early moieties (monofunctional PEGs; mPEGs) being linear with molecular weights of 12kD or less, and later moieties being of increased molecular weights. PEG2, a recent innovation in PEG technology, involves the coupling of a 30kD (or less) mPEG to lysine that is further reacted to form a branched structure that behaves like a linear mPEG of much larger molecular weight. These compounds are pH and temperature stable, and this factor along with the large molecular weight may account for the restricted volume of distribution seen with drugs utilising these reagents.

Three PEG-protein conjugates are currently approved for clinical use in the US, with more under clinical development. Pegademase is used in the treatment of severe combined immunodeficiency disease, pegaspargase for the treatment of various leukaemias, and pegylated interferon-α for chronic hepatitis C virus infections. As illustrated in the case of the 2 pegylated interferon-αs, all pegylated proteins are not equal. The choice of PEG reagent and coupling chemistry is critical to the properties of the PEG-protein conjugate, with the molecular weight of the moiety affecting its rate and route of clearance from the body, and coupling chemistry affecting the strength of the covalent attachment of PEG to therapeutic protein.

1. Introduction to Protein Pegylation: Properties of Poly(ethylene glycol) (PEG) Moieties

Pegylation was developed in the 1970s by Abuchowski and colleagues[1] to enhance the delivery of therapeutic proteins. The changes in pharmacokinetics and pharmacodynamics produced by pegylation have prompted research on the use of PEG-protein conjugates in several therapeutic areas, including oncology and infection.

Both protein and nonprotein molecules can be pegylated, although research efforts have focused primarily on PEG-protein conjugates. Pegylation, by increasing the molecular weight (MW) of the protein, typically reduces its excretion, permitting a decrease in dosage frequency. Thermal and pH stability is enhanced, helping to increase the termi-

nal half-life of the protein. By forming a 'shell' around the protein, pegylation can also decrease the immmunogenicity of the protein an protect it from proteolytic degredation. Drug toxicity is reduced, presumably as a result of the reduction in plasma concentration fluctuations induced by multiple daily administrateions.

The specific characteristics of poly(ethylene glycol) [PEG] moieties relevant to pharmaceutical applications are:

- water solubility
- high mobility in solution
- lack of toxicity
- lack of immunogenicity
- ready clearance from the body
- altered distribution in the body.[2,3]

Studies of PEG moieties in solution have shown that each ethylene oxide unit is tightly associated with 2 to 3 water molecules. This hydration, coupled with the rapid motion of the PEG molecule in aqueous medium, gives the PEG molecule the solubility properties of a molecule 5 to 10 times as large as a soluble protein of comparable MW. This size effect is readily revealed by several techniques such as size exclusion chromatography and gel electrophoresis.

The lack of toxicity of PEG has been established through many years of experience in the food, cosmetic and pharmaceutical industries.[4] The observed low immunogenicity of PEG is supported by several studies (reviewed by Harris[2,3]). A point of interest is the recent demonstration of the generation of anti-PEG antibodies under extreme conditions in rabbits.[5] This immune response, however, is not considered to be clinically significant, as there are no known examples of antibodies against PEG being generated from injection of therapeutic proteins into humans. The clearance of PEG moieties of differing MWs has been studied. Results show that the PEG moiety is cleared, without structural change, with clearance being proportional to the MW. Below a MW of about 20kD, the molecule is cleared via the kidney and excreted in the urine. Higher MW PEGs are cleared more slowly through the kidney, with liver clearance increasing as the PEG MW increases.[6]

Many properties of the native polymer are transferred to PEG-protein conjugates, which therefore exhibit a variety of desirable characteristics, some of which are mentioned above. All of these effects are dependent on the MW of the attached PEG moiety. The effect of PEG moiety attachment on solubility can be dramatic and is frequently observed with small molecule pharmaceuticals. The shielding ability of a covalently attached PEG moiety can lead to reduction or elimination of protein immunogenicity.[1,7] Unfortunately, insufficient knowledge of protein structure or the inability to selectively place the PEG moiety may hinder attempts to eliminate protein immunogenicity. The general approach is to attach the PEG moiety to some of the available lysines on the protein surface and hope that the desired result is achieved. Similarly, the shielding effect of the PEG moiety leads to reduced sensitivity to proteolysis for PEG proteins, and this effect is especially strong when high MW, branched PEG moieties are used.[8] Attachment of PEG moieties to a small protein will lead to a larger PEG-protein conjugate with a reduced rate of renal clearance. In sum, these properties enhance the circulation half-lives of proteins. PEG proteins also exhibit altered distribution in the body,[9] and when injected subcutaneously exhibit absorption into the bloodstream that is dependent on the MW of the PEG moieties. PEG proteins have enhanced stability that leads to a longer shelf life.

2. Chemistry for Pegylation

To couple a PEG moiety to a protein it is first necessary to activate the polymer by converting the hydroxyl terminus to some functional group capable of reacting with the functional groups found on the surface of proteins. The most common method has been to activate the PEG moiety with functional groups suitable for reaction with lysine and N-terminal amino groups. The PEG used is typically monofunctional PEG (mPEG) because this polymer resists cross-linking and aggregation. One early example of this approach is the reaction of mPEG tresylate with alkaline phosphatase [fig. 1].[10]

Several lysine residues are typically substituted

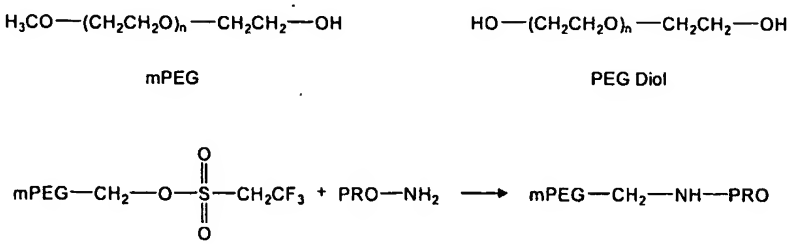


Fig. 1. Reaction of mPEG tresylate with alkaline phosphatase (represented as PRO-NH2).

during these reactions and each molecule of the protein can have a different pattern of lysine substitution. PEG moieties are polydisperse, which means that there is a distribution of MWs about the mean. The usual measure of polydispersity is the ratio of weight average MW to number average MW (Mw/Mn), with a larger ratio indicating a broader distribution. Compared with many polymers, PEG moieties have an unusually narrow polydispersity that is typically less than 1.05, and frequently close to 1.01; higher MWs typically have broader distributions.

The heterogeneity in lysine substitution and in PEG MWs is of some importance for PEG protein pharmaceuticals, and it is generally necessary to demonstrate that the pattern for a particular pharmaceutical can be measured and reproduced. It is possible in some instances, however, to reduce or eliminate heterogeneity in the position of substitution.

A major problem faced in PEG chemistry is that mPEG-OH is often contaminated with PEG diol (HO-PEG-OH). Some manufacturers produce low-diol mPEG, but some of this impurity is always present. Diol content can range as high as 10 to 15%.[11] Activation of the diol leads to a difunctional contaminant that is associated with cross-linking and aggregation. Diol arises from the presence of water contamination during the base-catalysed polymerisation of ethylene oxide to form mPEG, and given that there is a lower concentration of initiator (methoxide) in preparation of high MW mPEGs, water contamination and diol formation is a more serious problem in this case. Also, because the diol chain can grow at each end, the contaminating diol typically has a higher average MW than the desired mPEG.

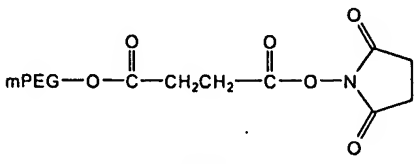
One characteristic of pegylation chemistry is that the diol and the resulting difunctional PEG are not removed. Consequently, one must be prepared to accept some protein cross-linking in the final product or make the effort to remove cross-linked product. An alternative is now available to purify the activated PEG reagent and remove the difunctional material. This route has the advantage of minimising the loss of expensive protein.

2.1 Monofunctional PEG (mPEG)

A wide range of PEG derivatives has been used for protein pegylation. Until recently these reagents were linear PEGs with MWs of 12kD or less. Examples of 2 PEG reagents are mPEG succinimidyl succinate (SS-PEG) and mPEG succinimidyl carbonate (SC-PEG) [fig. 2]. Although these reagents are widely used, including use in US Food and Drug Administration (FDA)-approved PEG-protein products, they are associated with several disadvantages including weak linkages between the PEG moiety and protein, potential unwanted side reactions, contamination, and restriction to low MW PEGs.

2.1.1 mPEG Succinimidyl Succinate

Activation of acids as succinimidyl esters has long been a popular chemistry, and SS-PEG has



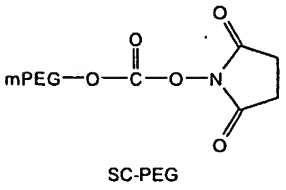


Fig. 2. Examples of two PEG reagents, mPEG succinimidyl succinate (SS-PEG) and succinimidyl carbonate (SC-PEG).

been used for many years.[12,13] The polymer is synthesised by reacting mPEG with succinic anhydride, followed by activation of the carboxylic acid as the succinimidyl ester. Contained in the structure of this polymer is a backbone that includes an ester linkage. This linkage remains after the mPEG is coupled to the protein (via a strong amide linkage) and is highly susceptible to hydrolysis after the polymer has been attached to the protein (fig. 3).

2.1.2 mPEG Succinimidyl Carbonate

SC-PEG is another widely used activated mPEG.[14] An advantage of this reagent relative to SS-PEG is that it does not contain a degradable ester linkage that can lead to the hydrolytic removal of the PEG moiety from the PEG-protein conjugate. The linkage formed between SC-PEG and a lysine residue is a urethane linkage (fig. 4). Urethanes formed with lysine groups are hydrolytically stable. It has recently been observed, however, that SC-PEG couples at a low pH to the histidine moieties of interferon (IFN)-α to form a hydrolytically unstable linkage.[15] This weak linkage can be used as an advantage in the preparation of a controlled release formulation with a mechanism similar to a prodrug, or it can be a disadvantage if stability during purification or storage is a concern. Another problem with SC-PEG is that it can undergo a Lossen rearrangement (the reaction of hydroxamic acids with water-soluble carbodiimides) during coupling to a protein.[16] This rearrangement can leave a chemical tag on the protein, resulting in protein-protein dimers.

The other problems typical of PEG chemistry are also found with SC-PEG. In particular, there is no mechanism for removing difunctional SC-PEG formed from the PEG diol contaminant. Since diol contamination is more prevalent with high MW mPEG, SC-PEG chemistry is restricted from a practical standpoint to low MW mPEGs such as mPEG 5000 and 12 000. This is a serious limitation, as there is now a great deal of interest in using higher MW mPEGs where it is only necessary to attach a single PEG moiety to a protein to achieve the desired effect of pegylation.

2.1.3 mPEG Propionaldehyde

More advanced forms of pegylation chemistry have been designed to avoid the problems of diol contamination, restriction to low MW mPEG, unstable linkages and lack of selectivity in substitution. One example is mPEG-propionaldehyde, which is easier to prepare and use than PEG-acetaldehyde

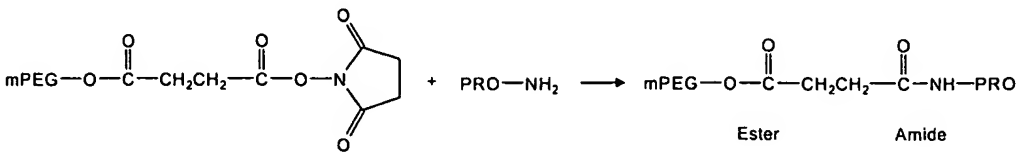


Fig. 3. Reaction of mPEG-succinimidyl succinate with a protein (represented as PRO-NH_2).

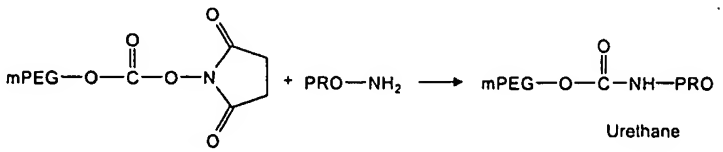


Fig. 4. The linkage formed between succinimidyl carbonate (SC) and a protein lysine is a urethane linkage.

because the acetaldehyde is very susceptible to dimerisation via aldol condensation. A key property of mPEG-propionaldehyde, as disclosed by Kinstler et al.[17] in work on pegylation of granulocyte colony-stimulating factor (G-CSF), is that the aldehyde is highly selective for the N-terminus of G-CSF. Although complete selectivity is not observed, the extensive heterogeneity normally seen with lysine chemistry is greatly reduced. Coupling of aldehyde proceeds through a Schiff base that is reduced *in situ* to give a stable secondary amine linkage (fig. 5).

Kinstler et al.[17] utilised mPEG 20 000 for coupling to G-CSF. Because of the size of G-CSF (18kD), this coupling resulted in an approximate doubling of the absolute MW of the molecule, and the heavy hydration and rapid motion of PEG resulted in an effective MW that is at least twice that of the native protein. It is expected that G-CSF with a single mPEG of 20kD would exhibit improved pharmacokinetic properties and enhanced efficacy relative to the native molecule. Clinical trials will ultimately determine the effects of pegylation on G-CSF. High MW PEG-propionaldehyde has also been used for pegylation of the tumour necrosis factor receptor for the treatment of rheumatoid arthritis.[18] In all these instances it was necessary to have a robust purification procedure to remove small amounts of contamination produced from PEG-diol-associated chemistry.

2.1.4 mPEG Vinylsulfone

Total selectivity in protein pegylation can be achieved by the introduction of a cysteine into the amino acid sequence of a protein by site-directed mutagenesis. In this way it is possible to position the mPEG in a location on the protein remote from the active or binding site. For example, mPEG vinylsulfone can be used to give selective conjugation with a cysteine group, with no lysine modification.[19] Another derivative that is useful for selective thiol pegylation is mPEG maleimide. Generally, this derivative is more reactive and easier to use than the mPEG vinylsulfone. Similarly, thiol pegylation can also be accomplished with orthopyridyldisulfide (OPSS) mPEG (OPSS-PEG). El Tayar et al.[20] recently prepared a highly active pegylated IFNβ using this derivative. In this case the available thiol was located in a sterically

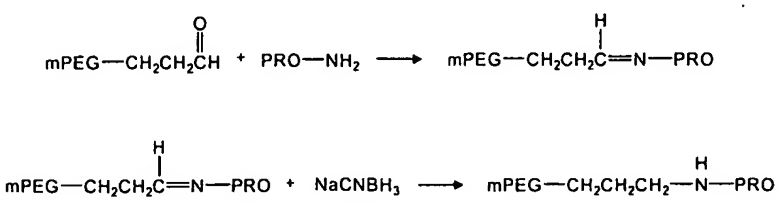


Fig. 5. Coupling of propionaldehyde proceeds through a Schiff base that is reduced *in situ* to give a stable se in the resulting mPEG-protein conjugate.

crowded region and little modification could be achieved with mPEG maleimide or mPEG vinylsulfone of high MW. The approach ultimately adopted was to couple a low MW di-OPSS-PEG (MW 2000) to the IFN and then couple a high MW PEG thiol to the remaining, terminal OPSS group.

2.1.5 mPEG Carboxylic Acid

The final hurdle to overcome with pegylation chemistry is removal of the difunctional derivative formed from diol contamination. This can be accomplished by preparing a mPEG carboxylic acid and purifying this acid by ion exchange chromatography.[21] By using this route it is possible to remove the di-acid formed from the diol. Also, we have found that there is some improvement in the polydispersity of the mPEG carboxylic acids because low MW PEG acids bind more tightly to the ion-exchange column than do the high MW PEG acids.

The first mPEG carboxylic acid that was produced was the succinate discussed earlier (see section 2.1.1). The ester linkage present in this molecule, however, leads to hydrolysis and degradation in an ion exchange column. Carboxymethylated PEG has been known for some time. Unfortunately, the succinimidyl ester of this compound is so reactive that it is difficult to use. To correct these problems, Harris and Kozlowski[21] prepared mPEG propionic acid, and then converted this compound into the succinimidyl active ester (SPA). This SPA-PEG compound has ideal reactivity and can be prepared with a diacid content of less than 1% for MW 20 000 PEG. Note that the starting mPEG for this product had about 5% of diol, but ion exchange chromatography of the intermediate acid reduced the difunctional material to less than 1%. SPA-PEG has been used for pegylation of human growth hormone receptor antagonist for the treatment of acromegaly. Clinical trials are complete and the PEG-protein conjugate is under regulatory review for marketing approval.[22-24]

2.2 PEG2

Another approach to preparing a pure, high MW, monofunctional PEG moiety is to couple crude mPEG-benzotriazole carbonate (BTC), of a MW up to 30kD, to lysine. This yields a high MW PEG acid with no hydrolytically degradable linkages. The crude PEG2 acid is contaminated with unreacted mPEG-BTC, 'PEG1', in which only a single mPEG is coupled to lysine, and the 'PEG3' that is formed from the reaction of activated PEG diol (or BTC-PEG-BTC) with 2 lysines followed by the coupling of 2 mPEG-BTC molecules to the remaining 2 amino groups (thus PEG3 is a diacid). During aqueous work-up, the unreacted mPEG-BTC is converted back to mPEG-OH, greatly simplifying subsequent ion exchange chromatography. Thus the reaction mixture contains a diacid, a zwitterion, a neutral mPEG and the desired monoacid. Careful ion exchange chromatography permits large-scale production of the desired pure PEG2-CO_2H. Activation of the succinimidyl ester (PEG2-NHS) is straightforward (fig. 6).

The PEG2 compound turns out to be a very exciting protein pegylation reagent that can be prepared in monofunctional form up to MW 60kD. Experiments have shown that PEG2, attached to a protein, 'acts' much larger than a corresponding linear mPEG of the same MW. This is due to the branching that occurs with the PEG2 moiety. Branched chain PEG-protein conjugates have increased pH and thermal stability compared with linear PEG conjugates.[8] Furthermore, the branched

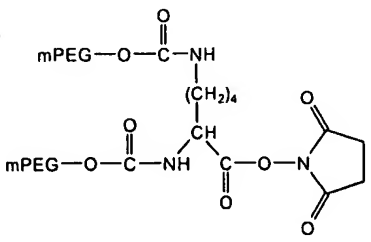


Fig. 6. PEG2-NHS is a pure, high molecular weight (MW), monofunctional PEG moiety created by coupling mPEG-benzotriazole carbonate of MW up to 30 kD to lysine.

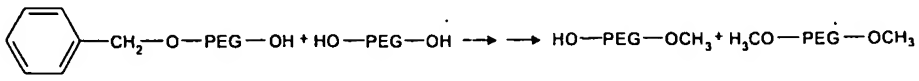


Fig. 7. Benzyloxy-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus, the diol is converted to the inert dimethyl ether.

structure of the PEG moiety may contribute to the restricted volume of distribution observed with PEG molecules. The availability of the very large PEG2-NHS means that only a single point of attachment is required to achieve the desired pegylation effect.[25] In addition, the pure PEG2-NHS can be converted into a range of other pure, monofunctional PEG derivatives including aldehyde and maleimide compounds that previously were always contaminated with difunctional material.

Another approach to the solution of the diol problem has recently been demonstrated by Bentley et al.[26] In this work, a crude PEG benzyloxy, containing diol impurity, was methylated and then hydrogenated to remove the benzyl group. Thus, the diol was converted to the inert dimethyl ether, which could be removed after activation and protein attachment (fig. 7).

3. Pharmaceutical PEGs

3.1 Pegademase

In light of the properties described above, several PEG-protein conjugates are been approved for clinical use by the US FDA or are currently under clinical development. Pegademase was approved in 1990 for use in severe combined immunodeficiency disease (SCID), a disease associated with an inherited deficiency of adenosine deaminase (ADA).[27] Before the availability of pegademase, patients with SCID were treated with partial exchange transfusions of red blood cells that contain ADA. These transfusions placed patients at risk for iron overload and transfusion-related viral infections.[28,29] Pegademase has approximately 1800-fold more ADA activity per millilitre than red blood cells; therefore, the drug produces higher concentrations of ADA activity than partial exchange transfusion.[30]

3.2 Pegaspargase

Pegaspargase is available for the treatment of acute lymphocytic leukaemia, acute lymphoblastic leukaemia and chronic myelogenous leukaemia. The native asparaginase compound is associated with a high incidence of allergic reactions and the development of neutralising antibodies that shorten its half-life, making it difficult to maintain effective plasma concentrations.[31] Pegaspargase reduces the tendency of the enzyme to induce an immune response, thus allowing patients with hypersensitivity to the native enzyme to tolerate pegaspargase without further incident.[32]

3.3 Other Therapeutic PEG Conjugates in Development

There are several important PEG proteins in advanced stage clinical trials, and it is expected that some of these proteins will receive approval within the next year. Included in this list of probable approvals are PEG-growth hormone receptor antagonist for treatment of acromegaly,[33] free radical scavengers, blood derivatives, antineoplastic agents, cardiovascular agents and antigens.[28]

Further evidence of the suitability of PEG-protein conjugates for human therapeutics is given by the FDA approval of 2 other PEG-containing products: stealth PEG-liposomes for delivery of doxorubicin for the treatment of Kaposi's sarcoma

(pegylated liposomal doxorubicin; Doxil®[1]) and PEG-lactide-glycolide-acrylate (FocalSeal®) used as a sealant in lung surgery.

3.4 Pegylated Interferon-α

Chronic hepatitis C virus (HCV) infection is a rapidly emerging disease that is the leading cause of liver cirrhosis and hepatocellular carcinoma.[34] The protein IFNα has been used extensively to treat HCV; however, monotherapy with IFN is associated with a sustained response in only a small percentage of patients.[34,35] This poor response is probably the result of multiple host (e.g. viral load) and viral factors (e.g. HCV genotype) as well as the inadequate pharmacokinetics of IFN. In particular, serum concentrations of IFN fall below the limit of detection within 24 hours of a single subcutaneous dose. Thus, even though the current treatment regimen calls for subcutaneous injection of IFN 3 times weekly, the virus has extended periods during which it is not exposed to a sustained therapeutic concentration of the drug. The addition of ribavirin to IFN in the treatment of HCV has increased the number of patients achieving a sustained virological response (38 to 43%).[35,36] This increase in response, however, is accompanied by a higher incidence of adverse events, some of which lead to discontinuation of therapy.

Pegylation of IFN improves the pharmacokinetics and, as a result, the pharmacodynamics of the drug, most likely leading to an increase in response. In 1999, clinical trials were conducted to evaluate the efficacy of IFNα-2a modified by attachment of mPEG of MW 5kD in patients with chronic HCV. Once weekly administration of PEG(5kD) IFNα-2a at doses of 15, 45, 90 and 135μg did not show improved efficacy over unmodified IFNα-2a 3 million international units (MIU) 3 times weekly.[37] More recently, clinical trials have evaluated a pegylated IFNα-2a prepared by coupling a branched PEG of MW 40kD to IFNα-2a.[38] The results of these trials are highly encouraging.[39,40] Additionally, a linear pegylated IFNα-2b of 12kD is currently approved in the European Union and the US for the treatment of HCV.

Further developement of pegylated IFNα-2a has involved examination of various pegylation chemistries. A detailed report of these studies will be published elsewhere.[41] After investigation, the moiety chosen for coupling with IFNα-2a was PEG2-NHS 40 000. Since the protein has a MW of about 19kD, the conjugate is a much larger molecule. Preclinical studies and modelling indicated that this conjugate, PEG(40kD) IFNα-2a would have desirable pharmacokinetics and activity. In particular, it was predicted that the steady-state blood concentration would be essentially constant (fig. 8). In practice, the desired result was achieved, and nearly constant blood concentrations are attained with once weekly subcutaneous injections.[42] Pegylation with PEG2-NHS 40 000 reduces renal clearance 100-fold relative to native IFNα-2a, and gives an elimination half-life of 77 *vs* 9 hours. PEG(40kD) IFNα-2a exhibits sustained absorption and a restricted volume of distribution when administered subcutaneously. This allows for a more convenient administration schedule (once weekly *vs* 3 times weekly for unmodified IFNα-2a).

Clinical results are now available for PEG (40kD) IFNα-2a. An open-label, parallel dose, global phase III trial randomised 531 patients to receive either PEG(40kD) IFNα-2a 180μg once weekly for 48 weeks (n = 267) or unmodified IFNα-2a 6 MIU 3 times weekly for 12 weeks followed by 3 MIU 3 times weekly for 36 weeks (n = 264). Results showed that PEG(40kD) IFNα-2a is associated with a greater virological response at week 48 (69 *vs* 28%, p = 0.001) and sustained virological response at week 72 (39 *vs* 19%, p = 0.001) compared with unmodified IFNα-2a.[40] Additionally, an open-label randomised, parallel dose study was performed in 271 patients with chronic HCV and biopsy-proven cirrhosis or bridging fibrosis. Patients were randomised to receive 48 weeks of treatment with IFNα-2a 3 MIU 3 times weekly

1 Use of tradenames is for product identification only and does not imply endorsement.

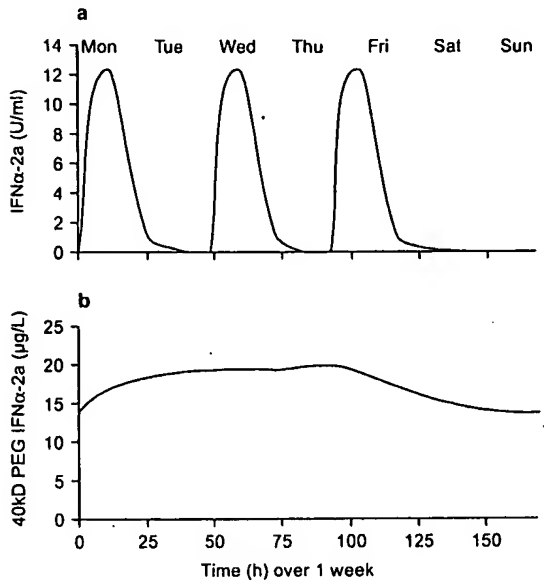


Fig. 8. Pharmacokinetic profiles for interferon (IFN)-α-2a and PEG(40kD)-IFNα-2a.

(n = 88) or PEG(40kD) IFNα-2a 90μg (n = 96) or 180μg (n = 87) once weekly followed by a 24-week treatment-free period. 44% of patients who were treated with 180μg PEG(40kD) IFNα-2a had a virological response at the end of 48 weeks' treatment (p = 0.001 *vs* IFNα-2a), and 30% achieved a sustained virological response at 72 weeks (p = 0.001 *vs* IFNα-2a). Among the 88 patients who received IFNα-2a 3 MIU 3 times weekly, only 14% achieved a virological response at 48 weeks and 8% had a sustained virological response at 72 weeks.[39]

It is well known that combination therapy with IFN and the antiviral agent ribavirin leads to enhanced efficacy compared with IFN alone.[34-36] Thus, it is of interest to combine PEG(40kD) IFNα-2a with ribavirin in the hope that improved results will be seen. Clinical trials to address this point are currently under way and the results of a small pilot study on PEG(40kD) IFNα-2a plus ribavirin in 20 patients have been announced.[43] Sustained virological and biochemical responses were observed in 9 of 20 patients. Five of 16 patients infected with HCV genotype 1 achieved a sustained virological response, as well as all 4 patients infected with other HCV genotypes.

A pegylated form of IFNα-2b has also been approved by the FDA.[44,45] Pegylation technology for preparation of a linear 12kD form of pegylated IFNα-2b with mPEG-SC 12 000 has been patented (PEG interferon-α-2b).[15] A special feature of this work, as noted above, is that pegylation at low pH (around pH 5) leads to extensive coupling to histidine via a hydrolytically unstable linkage.

Results from a phase III clinical trial comparing pegylated IFNα-2b once weekly with unmodified IFNα-2b 3 times weekly[45] showed that at the optimal dose (1.0 μg/kg), pegylated IFNα-2b gave sustained HCV clearance in 25% of 297 patients, whereas 12% of 303 patients who received native IFNα-2b showed sustained viral clearance ($p < 0.001$).

Results were recently reported for a phase III trial involving 1530 patients who were randomised to receive either pegylated IFNα-2b 1.5 μg/kg once weekly plus ribavirin 800 mg/day for 48 weeks (PEGα-2b 1.5/R); pegylated IFNα-2b 1.5 μg/kg once weekly plus ribavirin 1000 to 1200 mg/day for 4 weeks followed by pegylated IFNα-2b 0.5 μg/kg once weekly plus ribavirin 1000 to 1200 mg/day for 44 weeks (PEGα-2b 0.5/R); or IFNα-2b 3 MIU 3 times weekly plus ribavirin 1000 to 1200 mg/day for 48 weeks. A 54% sustained virological response was found overall in the pegylated IFNα-2b 1.5/R group compared with 47% in the IFNα-2b/ribavirin group (p = 0.01 *vs* interferon-α-2b + ribavirin), whereas 42% (p = 0.02 *vs* IFNα-2b/ribavirin) and 82% of patients in the PEGα-2b 1.5/R group with HCV genotype 1 and non-1, respectively, achieved a sustained virological response.[46] It is important to note, however, the difference between the highest response rate with pegylated IFNα-2b and IFNα-2b/ribavirin was only 7%.

Based on available data, it is obvious that both pegylated IFNα-2b and PEG(40kD) IFNα-2a have

superior efficacy when compared with unmodified IFNα-2b and IFNα-2a, respectively. The higher MW, branched pegylated IFNα-2a may have a better pharmacokinetic and pharmacodynamic profile than the lower MW pegylated IFNα-2b, offering a true once weekly administration regimen with the ability to maintain adequate serum IFN concentrations and possibly achieving improved sustained responses even in more difficult-to-treat patients. Confirmation of this supposition will require a head-to-head clinical trial between PEG(40kD) IFNα-2a and pegylated IFNα-2b. Clearly, pegylation of therapeutic proteins in general is valuable, and pegylation of IFN promises to increase treatment success in a growing population of patients diagnosed with HCV infection.

Acknowledgements

The development of this manuscript was supported by Shearwater Corporation. Shearwater Corporation manufactures the PEG reagents used in much of the work described in this review.

References

1. Abuchowski A, van Es T, Palczuk NC, et al. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem 1977; 252: 3578-81
2. Harris JM, Zalipsky S, editors. Poly(ethylene glycol): chemistry and biological applications. Washington, DC: American Chemical Society, 1997
3. Harris JM. Polyethylene glycol chemistry. Biotechnical and biomedical applications. New York: Plenum, 1992
4. Working PK, Newman MS, Johnson J, et al. Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives. In: Harris JM, Zalipsky S, editors. Poly(ethylene glycol): chemistry and biological applications. Washington, DC: American Chemical Society, 1997: 45-59
5. Cheng TL, Wu PY, Wu MF, et al. Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. Bioconjug Chem 1999; 10: 520-8
6. Yamaoka T, Tabata Y, Ikada Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. J Pharm Sci 1994; 83: 601-6
7. Katre NV. Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol. J Immunol 1990; 144: 209-13
8. Monfardini C, Schiavon O, Caliceti P, et al. A branched monomethoxypoly(ethylene glycol) for protein modification. Bioconjug Chem 1995; 6: 62-9
9. Caliceti P, Schiavon O, Veronese FM. Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers. Bioconjug Chem 1999; 10: 638-46
10. Yoshinaga Y, Harris JM. Effects of coupling chemistry on activity of a polyethylene glycol-modified enzyme. J Bioact Comp Polym 1989; 4: 17-24
11. Dust JM, Fang ZH, Harris JM. Proton NMR characterization of polyethylene glycols and derivatives. Macromolecules 1990; 23: 3742-6
12. Abuchowski A, Kazo GM, Verhoest CR, et al. Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates. Cancer Biochem Biophys 1984; 7: 175-86
13. Harris JM. Synthesis of polyethylene glycol derivatives. J Macromol Sci Rev Macromol Chem Phys 1985; C25: 325-73
14. Zalipsky S, Seltzer R, Menon-Rudolph S. Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. Biotechnol Appl Biochem 1992; 15: 100-14
15. Gilbert CW, Park-Cho M. Interferon polymer conjugates. US Patent 5,951,974, Sep. 14, 1999
16. Zalipsky S. Alkyl succinimidyl carbonates undergo Lossen rearrangement in basic buffers. Chem Commun 1998; 69-70
17. Kinstler OB, Brems DN, Lauren SL, et al. Characterization and stability of N-terminally PEGylated rhG-CSF. Pharm Res 1996; 13 (7): 996-1002
18. Edwards CK. Pegylated recombinant human soluble tumor necrosis factor receptor type I (rHu-sTNF-RI): novel high-affinity TNF receptor designed for chronic inflammatory diseases. Ann Rheum Dis 1999; 58 Suppl. 1: 173-81
19. Morpurgo M, Veronese FM, Kachensky D, et al. Preparation and characterization of poly(ethylene glycol) vinylsulfone. Bioconjug Chem 1996; 7: 363-8
20. El Tayar N, Roberts MJ, Harris JM, et al. Polyol-IFN-beta conjugates, WO 99/55377
21. Harris JM, Kozlowski A. Polyethylene glycol and related polymers monosubstituted with propionic or butanoic acids and functional derivatives thereof for biotechnical applications. US Patent 5,672,662, Sep 30; 1997
22. Thorner MO, Strasburger CJ, Wu Z, et al. Growth hormone (GH) receptor blockade with a PEG-modified GH (B2036-PEG) lowers serum insulin-like growth factor-I but does not acutely stimulate serum GH. J Clin Endocrinol Metab 1999 Jun; 84 (6): 2098-103
23. Goffin V, Bernichtein S, Carriere O, et al. The human growth hormone antagonist B2036 does not interact with the prolactin receptor. Endocrinology 1999 Aug; 140 (8): 3853-6
24. Ross RJ, Leung KC, Maamra M, et al. Binding and functional studies with the growth hormone receptor antagonist, B2036-PEG (pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. J Clin Endocrinol Metab 2001 Apr; 86 (4): 1716-23
25. Chapman, AP, Antoniw P, Spitali M, et al. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat Biotechnol 1999; 17: 780-3
26. Bentley MD, Harris JM, Kozlowski A. Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation. PCT US99/23536: Oct 8; 1999
27. Hershfield MS. Biochemistry and immunology of poly(ethylene glycol)-modified adenosine deaminase (PEG-ADA). In: Harris JM, Zalipsky S, editors. Poly(ethylene glycol): chemistry and biological applications. Washington, DC: American Chemical Society, 1997: 145-54
28. Burnham NL. Polymers for delivering peptides and proteins. Am J Hosp Pharm 1994; 51: 210-8
29. Hillman BC, Sorensen RU. Management options: SCIDS with adenosine deaminase deficiency. Ann Allergy 1994; 72: 395-404

30. Hershfield MS. PEG-ADA replacement therapy for adenosine deaminase deficiency: an update after 8.5 years. Clin Immunol Immunopathol 1995; 76: S228-32
31. Keating MJ, Holmes R, Lerner S, et al. L-asparaginase and PEG asparaginase – past, present, and future. Leuk Lymphoma 1993; 10 Suppl.: 153-7
32. Holle LM. Pegaspargase: an alternative. Ann Pharmacother 1997; 3: 616-24
33. Olson K, Gehant R, Mukku V, et al. Preparation and characterization of poly(ethylene glycol)ylated human growth hormone antagonist. In: Harris JM, Zalipsky S, editors. Poly (ethylene glycol): chemistry and biological applications. Washington, DC: American Chemical Society, 1997: 170-81
34. Liang JT, Rehermann B, Seeff LB, et al. Pathogenesis, natural history, treatment and prevention of hepatitis C. Ann Intern Med 2000; 132: 296-305
35. McHutchison JG, Gordon SC, Schiff ER, et al, and the Hepatitis Interventional Therapy Group. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 1998: 339: 1485-92
36. Poynard T, Marcellin P, Lee S, et al. Randomised trial of interferon alfa-2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alfa-2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998; 352: 1426-32
37. O'Brien C, Pockros P, Reddy R, et al. A double-blind, multicenter, randomized, parallel dose-comparison study of six regimens of 5kD, linear peginterferon alfa-2a compared with Roferon-A in patients with chronic hepatitis C. Antiviral Therapy 1999; 4 Suppl. 4: 15
38. Bailon P, Spence C, Schaffer CA, et al. Pharmacological properties of five polyethylene glycol conjugates of interferon alfa-2a. Antiviral Therapy 1999; 4 Suppl. 4: 27
39. Heathcote EJ, Shiffman ML, Cooksley GE, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med 2000; 343: 1673-80
40. Zeuzem S, Feinman SV, Rasenack J, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 2000; 343: 1666-72
41. Bailon P, Palleroni A, Schaffer CA, et al. Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem 2001 Mar-Apr; 12 (2): 195-202
42. Algranati NE, Sy S, Modi MW. A branched methoxy 40 kDa polyethylene glycol (PEG) moiety optimizes the pharmacokinetics (PK) of peginterferon α-2a (PEG-IFN) and may explain its enhanced efficacy in chronic Hepatitis C (CHC). Hepatology 1999; 30 (4 Pt 2): 190A
43. Sulkowski MS, Reindollar R, Yu J. Pegylated interferon alfa-2a (PEGASYS™) and ribavirin combination therapy for chronic hepatitis C: a phase II open-label study. Gastroenterology 2000; 118 Suppl. 2: 950
44. Glue P, Fang JW, Rouzier-Panis R, et al. Pegylated interferon-alpha2b; pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group. Clin Pharmacol Ther 2000 Nov; 68 (5): 556-67
45. Trepo C, Lindsay K, Niederau C, et al. Pegylated interferon alfa-2b (PEG-Intron) monotherapy is superior to interferon alfa-2b (Intron A) for the treatment of chronic hepatitis C [abstract GS2/07]. J Hepatology 2000; 32: 29
46. Manns MP, McHutchison JG, Gordon S, et al. Peginterferon alfa-2b plus ribavirin compared to interferon alfa-2b plus ribavirin for the treatment of chronic hepatitis C: 24 week treatment analysis of a multicenter, multinational phase III randomized controlled trial. Hepatology 2000; 32 (4): 297A

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48 — A2

(11) International Publication Number: WO 99/55377

(43) International Publication Date: 4 November 1999 (04.11.99)

(21) International Application Number: PCT/US99/09161

(22) International Filing Date: 28 April 1999 (28.04.99)

(30) Priority Data:
60/083,339 28 April 1998 (28.04.98) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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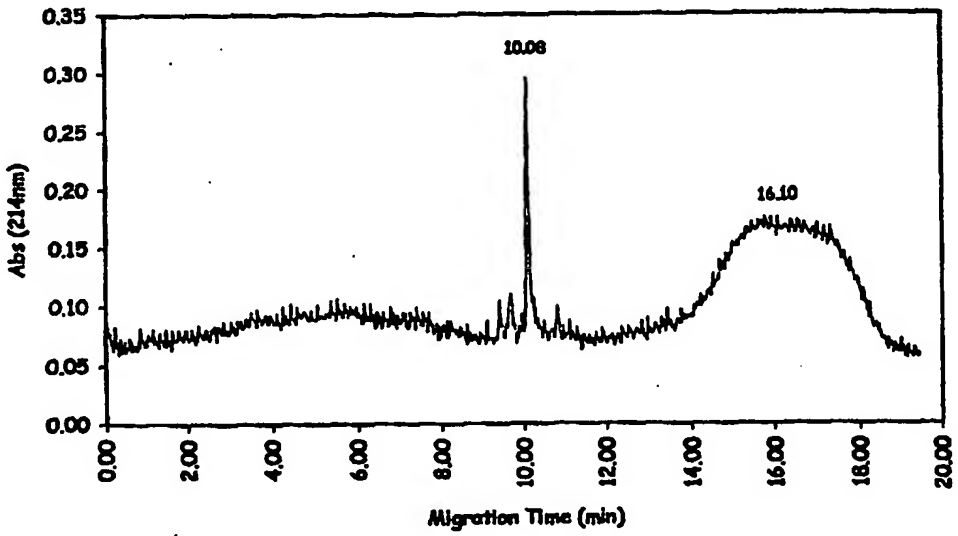

(57) Abstract

PEG–IFN–β conjugates, where a PEG moiety is covalently bound to Cys^{17} of human IFN–β, are produced by a process of site specific PEGylation with a thiol reactive PEGylating agent. A pharmaceutical composition and a method for treating infections, tumors and autoimmune and inflammatory diseases are also provided. The invention further relates to a method for the stepwise attachment of PEG moieties in series to a polypeptide, and more particularly to IFN–β.

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CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

POLYOL-IFN-BETA CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority under 35 U.S.C. §119(e) from U.S. provisional application no. 60/083,339, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to polyol-IFN-β conjugates wherein a polyol unit is covalently bound to Cys^{17}. Further objects of the present invention are the process for their site-specific production as well as their use in the therapy, prognosis or diagnosis of bacterial infections, viral infections, autoimmune diseases and inflammatory diseases. The present invention further relates to a method for the stepwise attachment of two or more PEG moieties to a polypeptide.

BACKGROUND OF THE INVENTION

Human fibroblast interferon (IFN-β) has antiviral activity and can also stimulate natural killer cells against neoplastic cells. It is a polypeptide of about 20,000 Da induced by viruses and double-stranded RNAs. From the nucleotide sequence of the gene for fibroblast interferon, cloned by recombinant DNA technology, Derynk et al. (Nature, 285:542-547, 1980) deduced the complete amino acid sequence of the protein. It is 166 amino acid long.

Shepard et al. (Nature, 294:563-565, 1981) described a mutation at base 842 (Cys → Tyr at position 141) that abolished its anti-viral activity, and a variant clone with a deletion of nucleotides 1119-1121.

Mark et al. (Proc. Natl. Acad. Sci. U.S.A., 81(18):5662-5666, 1984) inserted an artificial mutation by replacing base 469 (T) with (A) causing an amino acid switch from Cys → Ser at position 17. The resulting IFN-β was reported to be as active as the 'native' IFN-β and stable during long-term storage (-70°C).

Covalent attachment of the hydrophilic polymer

polyethylene glycol, (PEG), also known as polyethylene oxide, (PEO), to molecules has important applications in biotechnology and medicine. In its most common form, PEG is a linear polymer having hydroxyl groups at each terminus:

$$HO{-}CH_2{-}CH_2O(CH_2CH_2O)_nCH_2CH_2{-}OH$$

This formula can be represented in brief as HO-PEG-OH, where it is meant that -PEG- represents the polymer backbone without the terminal groups:

"-PEG-" means "$-CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2-$"

PEG is commonly used as methoxy-PEG-OH, (m-PEG), in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to chemical modification.

$$CH_3O{-}(CH_2CH_2O)_n{-}CH_2CH_2{-}OH$$

Branched PEGs are also in common use. The branched PEGs can be represented as $R(\text{-PEG-OH})_m$ in which R represents a central core moiety such as pentaerythritol or glycerol, and m represents the number of branching arms. The number of branching arms (m) can range from three to a hundred or more. The hydroxyl groups are subject to chemical modification.

Another branched form, such as that described in PCT patent application WO 96/21469, has a single terminus that is subject to chemical modification. This type of PEG can be represented as $(CH_3O{-}PEG{-})_pR{-}X$, whereby p equals 2 or 3, R represents a central core such as lysine or glycerol, and X represents a functional group such as carboxyl that is subject to chemical activation. Yet another branched form, the "pendant PEG", has reactive groups, such as carboxyl, along the PEG backbone rather than at the end of PEG chains.

In addition to these forms of PEG, the polymer can also be prepared with weak or degradable linkages in the backbone. For example, Harris has shown in U.S. Patent Application 06/026,716 that PEG can be prepared with ester linkages in the polymer backbone that are subject to hydrolysis. This hydrolysis results in cleavage of the polymer into fragments of lower molecular weight, according to the reaction scheme:

$$-PEG-CO_2-PEG- + H_2O \rightarrow -PEG-CO_2H + HO-PEG-$$

According to the present invention, the term polyethylene glycol or PEG is meant to comprise all the above described derivatives.

The copolymers of ethylene oxide and propylene oxide are closely related to PEG in their chemistry, and they can be used instead of PEG in many of its applications. They have the following general formula:

$$HO-CH_2CHRO(CH_2CHRO)_nCH_2CHR-OH$$

wherein R is H or CH_3.

PEG is a useful polymer having the property of high water solubility as well as high solubility in many organic solvents. PEG is also non-toxic and non-immunogenic. When PEG is chemically attached (PEGylation) to a water insoluble compound, the resulting conjugate generally is water soluble, as well as soluble in many organic solvents.

PEG-protein conjugates are currently being used in protein replacement therapies and for other therapeutic uses. For example, PEGylated adenosine deaminase (ADAGEN®) is being used to treat severe combined immunodeficiency disease (SCIDS), PEGylated L-asparaginase (ONCAPSPAR®) is being used to treat acute lymphoblastic leukemia (ALL), and PEGylated interferon-α (INTRON(R) A) is in Phase III trials for treating hepatitis C.

For a general review of PEG-protein conjugates with clinical efficacy see N.L. Burnham, Am. J. Hosp. Pharm., 15:210-218, 1994.

A variety of methods have been developed to PEGylate proteins. Attaching PEG to reactive groups found on the protein is typically done utilizing electrophilically activated PEG derivatives. Attaching PEG to the α- and ε-amino groups found on lysine residues and the N-terminus results in a conjugate consisting of a mixture of products.

Generally, such conjugates consist of a population of the several PEG molecules attached per protein molecule ("PEGmers") ranging from zero to the number of amino groups in the protein. For a protein molecule that has been singly modified, the PEG unit may be attached at a number of different amine sites.

This type of non-specific PEGylation has resulted in a number of conjugates that become almost inactive. Reduction of activity is typically caused by shielding the protein's active binding domain as is the case with many cytokines and antibodies. For example, Katre et al. in U.S. Patent 4,766,106 and U.S. Patent 4,917,888 describe the PEGylation of IFN-β and IL-2 with a large excess of methoxy-polyethylene glycolyl N-succinimidyl glutarate and methoxy-polyethylene glycolyl N-succinimidyl succinate. Both proteins were produced in microbial host cells, which allowed the site-specific mutation of the free cysteine to a serine. The mutation was necessary in microbial expression of IFN-β to facilitate protein folding. In particular, the IFN-β used in these experiments is the commercial product Betaseron®, in which Cys^{17} residue is replaced with a serine. Additionally, the absence of glycosylation reduced its solubility in aqueous solution. Non-specific PEGylation resulted in increased solubility, but a major problem was the reduced level of activity and yield.

European Patent Application EP 593 868, entitled PEG-Interferon Conjugates, describes the preparation of PEG-IFN-α conjugates. However, the PEGylation reaction is not site-specific, and therefore a mixture of positional isomers of PEG-IFN-α conjugates are obtained (see also Monkarsh et al., ACS Symp. Ser., 680:207-216, 1997).

Kinstler et al. in European Patent Application EP 675 201 demonstrated the selective modification of the N-terminal residue of megakaryocyte growth and development factor (MGDF) with mPEG-propionaldehyde. This allowed for reproducible PEGylation and pharmacokinetics from lot to lot. Gilbert et al. in U.S. Patent 5,711,944 demonstrated that PEGylation of IFN-α with an optimal level of activity could be produced. In this instance a laborious purification step was needed to obtain the optimal conjugate.

The majority of cytokines, as well as other proteins, do not possess a specific PEG attachment site and, apart from the examples mentioned above, it is very likely that some of the isomers produced through the PEGylation reaction be partially or

totally inactive, thus causing a loss of activity of the final mixture.

Site-specific mono-PEGylation is thus a desirable goal in the preparation of such protein conjugates.

Woghiren et al. in Bioconjugate Chem., 4(5):314-318, 1993, synthesized a thiol-selective PEG derivative for such a site-specific PEGylation. A stable thiol-protected PEG derivative in the form of an orthopyridyl disulfide reactive group was shown to specifically conjugate to the free cysteine in the protein, papain. The newly formed disulfide bond between papain and PEG could be cleaved under mild reducing conditions to regenerate the native protein.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicants at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

In the present invention, polyol-IFN-β conjugates, and particularly PEG-IFN-β conjugates, are provided wherein a polyol unit is covalently bound to Cys^{17}. The specific conjugation is obtained by allowing a thiol-reactive polyol agent to react with the Cys^{17} residue in IFN-β. Such conjugates are expected to show increased effectiveness *in vivo*. The aim is to obtain increased solubility at neutral pH, increased stability (decreased aggregation), decreased immunogenicity, and no loss of activity with respect to 'native' IFN-β. The results of such conjugation would decrease the number of doses for an intended effect, simplify and stabilize the formulation of a pharmaceutical composition, and possibly increase the long-term efficacy.

The present invention further provides a method for the stepwise attachment of PEG moieties in series to a polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the Capillary Electrophoresis (CE) graph of the PEG-IFN-β conjugate prior to purification.

Figures 2A-2C show the purification of the PEG-IFN-β conjugate carried out by size exclusion chromatography (Superose 12): Fig. 2A - first pass; Fig. 2B - second pass; Fig. 2C - third pass.

Figure 3 shows the SDS-PAGE chromatography of purified PEG-IFN-β conjugate from the third pass of chromatography. Lanes 1 and 4 are protein molecular weight standards, lane 2 is "native" IFN-β, and lane 3 is PEG-IFN-β conjugate.

Figure 4 reports the Capillary Electrophoresis (CE) graph of purified PEG-IFN-β conjugate in which IFN-β is PEGylated with mPEG-$OPSS_{5k}$.

Figure 5 reports the MALDI MS spectrum of purified PEG-IFN-β conjugate.

Figure 6 shows a comparison between the anti-viral activity of "native" IFN-β and of PEG-IFN-β conjugate. WISH cells were incubated with indicated concentrations of IFN-β samples for 24 hours prior to challenge with cytopathic dose of vesicular stomatitis virus. The cytopathic effect was determined after an additional 48 hours by MTT conversion.

Figure 7 shows the binding profile of IFN-β and PEG-IFN in Daudi cells.

Figure 8 shows the pharmokinetic profile of IFN-β and PEG-IFN in mice following intravenous administration. The dotted lines indicate assay LOQ for each standard curve.

Figure 9 shows the pharmokinetic profile of IFN-β and PEG-IFN in mice following subcutaneous administration. The dotted lines indicate assay LOQ for each standard curve.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the attachment of a polyol moiety, more specifically a PEG moiety, to the Cys^{17} residue of human IFN-β unexpectedly increased (or at least retained and did not result in a decrease) the IFN-β biological activity from that of native

human interferon-β. Thus, not only does IFN-β with a polyol moiety attached to the Cys^{17} residue exhibit the same or increased IFN-β biological activity but this polyol-IFN-β conjugate also provides the desirable properties conferred by the polyol moiety, such as increased solubility.

"IFN-β", as used herein, means human fibroblast interferon, as obtained by isolation from biological fluids or as obtained by DNA recombinant techniques from prokaryotic or eukaryotic host cells as well as its salts, functional derivatives, precursors and active fractions, provided that they contain the cysteine residue appearing at position 17 in the naturally occurring form.

The polyol moiety in the polyol-IFN-β conjugate according to the present invention can be any water-soluble mono- or bifunctional poly(alkylene oxide) having a linear or branched chain. Typically, the polyol is a poly(alkylene glycol) such as poly(ethylene glycol) (PEG). However, those of skill in the art will recognize that other polyols, such as, for example poly (propylene glycol) and copolymers of polyethylene glycol and polypropylene glycol, can be suitably used.

As used herein, the term "PEG moiety" is intended to include, but is not limited to, linear and branched PEG, methoxy PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, dendrimer PEG, copolymers of PEG and one or more polyols, and copolymers of PEG and PLGA (poly(lactic/glycolic acid)).

The definition "salts" as used herein refers both to salts of the carboxyl-groups and to the salts of the amino functions of the compound obtainable through known methods. The salts of the carboxyl-groups include inorganic salts as, for example, sodium, potassium, calcium salts and salts with organic bases as those formed with an amine as triethanolamine, arginine or lysine. The salts of the amino groups included for example, salts with inorganic acids as hydrochloric acid and with organic acids as acetic acid.

The definition "functional derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the terminal N- or C- groups according to known methods

and are included in the present invention when they are pharmaceutically acceptable, i.e., when they do not destroy the protein activity or do not impart toxicity to the pharmaceutical compositions containing them. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

The "precursors" are compounds which are converted into IFN-β in the human or animal body.

As "active fractions" of the protein, the present invention refers to any fragment or precursor of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example, residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same activity of IFN-β as medicament.

The conjugates of the present invention can be prepared by any of the methods known in the art. According to an embodiment of the invention, IFN-β is reacted with the PEGylating agent in a suitable solvent and the desired conjugate is isolated and purified, for example, by applying one or more chromatographic methods.

"Chromatographic method" means any technique that is used to separate the components of a mixture by their application on a support (stationary phase) through which a solvent (mobile phase) flows. The separation principles of the chromatography are based on the different physical nature of stationary and mobile phase.

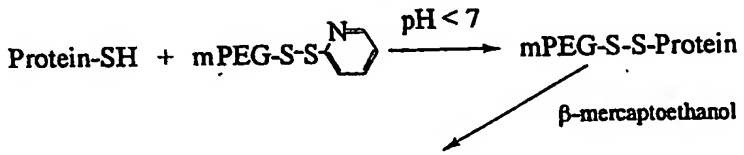
Some particular types of chromatographic methods, which are well-known in the literature, include: liquid, high pressure liquid, ion exchange, absorption, affinity, partition, hydrophobic, reversed phase, gel filtration, ultrafiltration or thin-layer chromatography.

The "thiol-reactive PEGylating agent", as used in the present application, means any PEG derivative which is capable of reacting with the thiol group of the cysteine residue. It can be, for example, PEG containing a functional group such as

orthopyridyl disulfide, vinylsulfone, maleimide, iodoacetimide, and others. According to a preferred embodiment of the present invention, the thiol-reactive PEGylating agent is the orthopyridyl disulfide (OPSS) derivative of PEG.

The PEGylating agent is used in its mono-methoxylated form where only one terminus is available for conjugation, or in a bifunctional form where both termini are available for conjugation, such as for example in forming a conjugate with two IFN-β covalently attached to a single PEG moiety. It has preferably a molecular weight between 500 and 100,000.

A typical reaction scheme for the preparation of the conjugates of the invention is presented below:



$$\text{Protein-S-H} + \text{mPEG-S-H} + \text{HOCH}_2\text{CH}_2\text{-S-S-CH}_2\text{CH}_2\text{OH}$$

The second line of the above scheme reports a method for cleaving the PEG-protein linkage. The mPEG-OPSS derivative is highly selective for free sulphydryl groups and reacts rapidly under acidic pH conditions where the IFN-β is stable. The high selectivity can be demonstrated from the reduction of the conjugate to the native form of IFN-β and PEG.

The disulfide bond that is produced between the protein and PEG moieties has been shown to be stable in the circulation, but it can be reduced upon entering the cell environment. Therefore it is expected that this conjugate, which does not enter the cell, will be stable in the circulation until it is cleared.

It should be noted that the above reaction is site-specific because the other two Cys residues appearing at positions 31 and 141 in the naturally occurring form of human IFN-β do not react with the thiol-reactive PEGylating agent since they form a disulfide bridge.

The present invention is also directed to a method for the stepwise attachment of two or more PEG moieties to a polypeptide. This method is based upon the recognition that a low molecular weight activated PEG reacts more completely with a sterically hindered reaction site on a protein than does a high molecular weight activated PEG. PEG-modification of expensive therapeutic proteins must be cost effective in order for the production of the PEG conjugate to be practical. In addition, in order to reduce glomerular filtration and optimize the pharmacological properties of the PEG-protein conjugate, the conjugate should have an effective size equivalent to that of a protein with a molecular weight of 70 kDa. This means that for a site specific modification where one PEG will be attached, a PEG derivative having a molecular weight of greater than 20 kDa is preferably attached. If the site of modification is sterically crowded, the reactive group on the large PEG moiety may have difficulty reaching the modification site and thus will lead to low yields. A preferred method of PEGylating a polypeptide according to the present invention increases the yield of site-specific PEGylation by first attaching a small hetero or homobifunctional PEG moiety that, due to its relatively smaller size, can react with sterically crowded sites. Subsequent attachment of a large molecular weight PEG derivative to the small PEG results in high yield of the desired PEGylated protein.

The method for stepwise attachment of two or more PEG moieties in series to a polypeptide according to the present invention includes attaching a low molecular weight heterbifunctional or homobifunctional PEG moiety first to the polypeptide and then attaching a monofunctional or bifunctional PEG moiety to the free terminus of the low molecular weight PEG moiety that is attached to the polypeptide. Following the stepwise attachment of two or more PEG moieties in series to a polypeptide, which polypeptide is preferably IFN-β and where Cys^{17}, located in a sterically crowded site, is the preferred site of PEG attachment, the PEG-polypeptide conjugate can be purified using one or more of the purification techniques such as ion exchange chromatography, size exclusion chromatography,

hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

The low molecular weight PEG moiety has the formula:

$$W\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}X,$$

where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide. W and X are preferably independently selected from orthopyridyl disulfide, maleimides, vinyl sulfones, iodoacetamides, amines, thiols, carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin. The low molecular weight PEG moiety preferably has a molecular weight in the range of about 100 to 5,000 daltons.

The monofunctional or bifunctional PEG moiety for attachment to the free terminus of a low molecular weight PEG that is attached to the polypeptide has preferably a molecular weight in the range of about 100 daltons to 200 kDa and is preferably a methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG. The monofunctional or bifunctional PEG furthermore has the formula:

$$Y\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}Z,$$

where Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety that is attached to the polypeptide and Z is $-OCH_3$ or a group reactive with to form a bifunctional conjugate.

The PEG-polypeptide conjugate produced by the above method for stepwise attachment of two or more PEG moieties can be used to produce a medicament or pharmaceutical composition for treating diseases or disorders for which the polypeptides is effective as an active ingredient.

Another object of the present invention is to provide the conjugates in substantially purified form in order for them to be suitable for use in pharmaceutical compositions, as active

ingredient for the treatment, diagnosis or prognosis of bacterial and viral infections as well as autoimmune, inflammatory diseases and tumors. Such pharmaceutical compositions represent a further object of the present invention.

Non-limiting examples of the above-mentioned diseases include: septic shock, AIDS, rheumatoid arthritis, lupus erythematosus and multiple sclerosis.

Further embodiments and advantages of the invention will be evident in the following description.

An embodiment of the invention is the administration of a pharmacologically active amount of the conjugates of the invention to subjects at risk of developing one of the diseases reported above or to subjects already showing such pathologies.

Any route of administration compatible with the active principle can be used. Parenteral administration, such as subcutaneous, intramuscular or intravenous injection is preferred. The dose of the active ingredient to be administered depends on the basis of the medical prescriptions according to age, weight and the individual response of the patient.

The dosage can be between 10 μg and 1 mg daily for an average body weight of 75 kg, and the preferable daily dose is between 20 μg and 200 μg.

The pharmaceutical composition for parenteral administration can be prepared in an injectable form comprising the active principle and a suitable vehicle. Vehicles for the parenteral administration are well known in the art and include, for example, water, saline solution, Ringer solution and/or dextrose. The vehicle can contain small amounts of excipients in order to maintain the stability and isotonicity of the pharmaceutical preparation. The preparation of the solutions can be carried out according to the ordinary modalities.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLE 1: Preparation of PEG-IFN-β Conjugate

Modification of IFN-β with $mPEG_{5K}$-OPSS

Recombinant human IFN-β, stable at a concentration of 0.37 mg/ml in 50 mM sodium acetate buffer, pH 3.6, was used for the preparation of a PEG-IFN-β conjugate. Approximately 1.0 ml of 6 M urea was added to 2 ml of IFN-β at a concentration of 0.37 mg/ml (0.74 mg, 3.7×1^{-8} moles). $mPEG_{5K}$-OPSS was added in a molar excess of 50 moles to one mole of IFN-β and the two were allowed to react in a polypropylene vial for either 2 hours at 37°C or 1 hour at 50°C. The reaction mixture was analyzed with Capillary Electrophoresis (CE) graph to determine the extent of PEG-IFN-β conjugate formation by the PEGylation reaction prior to any purification (Fig. 1). A typical yield for this reaction is 50% PEG-IFN-β. The reaction products were filtered from the reaction mixture with a 0.22 mm syringe filter and the filtered solution was then loaded onto a size exclusion column (either Superose 12 or Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. Fig. 2A shows the elution profile from the purification of the PEG-IFN-β conjugate on a Superose 12 size exclusion chromatography column. The peaks were collected and analyzed with SDS-PAGE (Fig. 3). The fractions containing the PEG-IFN-β conjugate were pooled together and concentrate was then reloaded to the same size exclusion column to further purify the PEG-IFN-β conjugate due to the close proximity of the "native" IFN-β peak (Fig. 2B). This procedure was repeated (third pass) to ensure purity (Fig. 2C). Fig. 4 and Fig. 5 show the Capillary Electrophoresis graph and the MALDI MS spectrum, respectively, of the purified PEG-IFN-β conjugate.

Modification of IFN-β with $mPEG_{30K}$-OPSS

Recombinant human IFN-β was provided is stable in solution at 0.36 mg/ml in 50 mM sodium acetate buffer, pH 3.6.

Approximately 36 mg of $mPEG_{30K}$-OPSS in 3 ml deionized H_2O was added to 3 ml of IFN-β at 0.36 mg/ml (1.08 mg, $4.9\text{-}x10^{-8}$ moles) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction mixture was analyzed with capillary electrophoresis for extent of modification. Typical yields for this reaction are <30%. The solution was then loaded onto a size exclusion column (Superose 12, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents.

EXAMPLE 2: Biological Activity of the PEG-IFN-β Conjugate

To assess the effects of PEGylation on the anti-viral activity of human recombinant IFN-β, human WISH amniotic cells were preincubated with either freshly prepared IFN-β (same lot as used for PEGylation) or PEG-IFN-β conjugate. The IFN-β-mediated anti-viral activity, as measured by the WISH-VSV cytopathicity assay, was determined according to an anti-viral WISH bioassay developed based on the protocol of Novick et al., J. Immunol., 129:2244-2247 (1982). The materials used in this WISH assay is as follows:

WISH cells (ATCC CCL 25)

Vesicular Stomatitis Virus stocks (ATCC V-520-001-522), stored at -70°C

IFN-β, human recombinant, InterPharm Laboratories LTD (32,075-type, Batch # 205035), 82 x 10^6 IU/ml, specific activity: 222 x 10^6 IU/mg

PEG-IFN-β conjugate as prepared in Example 1 and maintained in PBS, pH 7.4

WISH Growth medium (MEM high glucose with Earls salts + 10% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μg/ml)

WISH Assay medium (MEM high glucose with Earls salts + 5% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μg/ml)

MTT at 5 mg/ml in PBS, stored at minus 70°C.

The protocol for the WISH assay is as follows:

Dilute the IFN-β samples to 2X the starting concentration in WISH assay medium.

Make three-fold dilutions of IFN-β samples in WISH assay medium in flat-bottomed 96-well plate so that each well contains 50 μl of diluted IFN-β sample (some control wells receive 50 μl of WISH assay medium only).

Harvest log growth phase WISH cells with trypsin/EDTA solution, wash in WISH assay medium, and bring to a final concentration of 0.8×10^6 cells/ml.

Add 50 μl of WISH cell suspension (4×10^4 cells per well) to each well. Final concentration of IFN-β exposed to the cells is now 1X.

After incubation for 24 hours in a 5% CO_2 humidified incubator, 50 μl of a 1:10 dilution (in WISH assay medium) of VSV stock (a dose predetermined to lyse 100 percent of WISH cells within 48 hours) is added to all wells except for the no virus control wells (these receive an equal volume of assay medium only).

After an additional 48 hours, 25 μl of MTT solution is added to all wells, after which plates are incubated for an additional 2 hours in an incubator.

The contents of the wells are removed by plate inversion, and 200 μl of 100% ethanol is added to the wells.

After 1 hour, the plates are read at 595 nm using the Soft max Pro software package and Spectramax spectrophotometer system (Molecular Devices).

Table 1. Antiviral Activity of PEGylated and Mock-PEGylated IFN-beta Samples

IFN-beta Sample*	EC_{50}**
PEG-IFN-β conjugate	3.9 +/- 0.7 pg/ml
IFN-β	16.4 +/- 1.0 pg/ml

* Stock concentrations of IFN-β in samples determined by amino acid analysis.

** EC_{50} (+/- S.D.) was determined by software program Microcal Origin 4.1

As demonstrated in Fig. 6 and Table 1 above, the PEG-IFN-β conjugate maintained a level of anti-viral activity superior to that of the freshly prepared parental lot of IFN-β.

The observation that the PEG-IFN-β conjugate has approximately 4-fold higher bioactivity than that of freshly prepared IFN-β may be also a consequence of the increased stability of the PEG-IFN-β conjugate with respect to the "native" IFN-β after addition of WISH cell assay medium.

EXAMPLE 3: In Vitro Assays of the Relative Activity of PEG-IFN samples

Relative bioactivity of PEG[30 kD]-IFN-β and PEG[2 X 20 kD]-IFN-β was determined by WISH assay using the standard protocol described in Example 2 (Table 2). Three independent assays were performed by three different individuals at separate times.

Table 2. Relative antiviral activity of PEG-IFN-β

	Relative Interferon Activity* (from three studies)			
Sample	Assay 1	Assay 2	Assay 3	Average (S.D.)
PEG[30kD]-IFN-β	3.2 X higher	3.1 X higher	1.8 X higher	3.0 X (0.78) higher
PEG[2 x 20kD]-IFN-β	4.2 X higher	1.3 X higher	0.85 X higher	2.1 X (1.8) higher

*EC50 doses compared with standard IFN-β included in each assay.

**Comparison based on IFN-β concentration of 330 ug/ml. Stock concentrations of PEG[30 kD]-IFN-β (5.41 ug/ml) and PEG[2 x 20 kD]-IFN-β (6.86 ug/ml) were determined by AAA.

The binding of PEG-IFN-β to its receptor on cells was evaluated in the presence of a fixed amount of ^{125}I-IFN-α2a. IFN-α2a was radiolabeled with ^{125}I using the chloramine T method. The ^{125}I bound IFNα2a was removed from free iodine by running the reactants through a Sephadex G25 column and pooling the protein containing fractions (Pharmacia). ^{125}I-IFN-α2a was quantified by an IFN-α2a ELISA assay (Biosource, USA) and the specific activity was determined. Daudi cells grown in the exponential phase of growth were harvested and 2 x 10^6 cells were incubated with 0.5 nM ^{125}I-IFN-α2a for 3 hours at room temperature in the presence of different concentrations of PEG-IFN-β or IFN-α2a diluted in an assay buffer which is RPMI 1640 containing 2%

fetal bovine serum and 0.1% sodium azide. At the end of the incubation, the cells were spun through a layer of phthalate oil and the cell bound radioactivity was counted on the gamma counter. Furthermore, the binding of PEG[30kD]-IFN-β and PEG[2 x 20kD]-IFN-β to the receptor were very similar or close to the binding activity of IFN-β as shown in Fig. 7.

In addition, relative activity was determined in a Daudi cell (human B cell lymphoma) anti-proliferation assay (Table 3). All IFNs were made at a 2x concentration of 200 ng/ml. Samples were diluted three-fold down the length of the plate at a final volume of 100 ul. 1 x 10^5 cells/well (100 µls) were added to each well and incubated for a total of 72 hours at 37°C in CO_2 humidified incubator. After 48 hours, tritiated (^3H) thymidine were added at 1 µCi/well in 20 ul. At the end of the 72 hour incubation, the plate was harvested with the Tomtek Plate Harvester. The results shown in Table 3 indicate that no detectable loss of IFN activity was observed from PEGylation. In fact, the activity was found to be somewhat higher than free IFN-β. This may be due to the formation of inactive aggregates in the free IFN or to the differences in quantitation methods (amino acid analysis for PEG-IFN samples and RP-HPLC for IFN-β).

Table 3. Daudi Anti-Proliferation Assay

	IC_{50}dose*	Fold increase vs IFN
IFN-β (Plate 1)	1153.1	--
PEG[30kD]-IFN (71 A)	695.6	1.6X
IFN-β (Plate 2)	1005.8	--
PEG[40kD]-IFN (71 B)	629.4	1.7X

*pg/ml

EXAMPLE 4: Pharmacokinetic Studies in Mice

Intravenous Administration

Mice were injected with 100 ng of IFN-β, PEG[30kD]-IFN-β or PEG[2 X 20 kD]-IFN-β and blood was removed at indicated times thereafter. Serum concentrations of IFN-β were determined

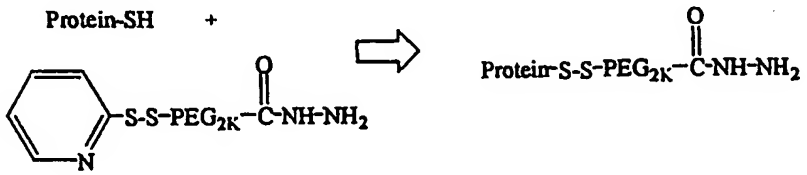
by IFN-β-specific ELIAS (Toray Industries) and the results are shown in Fig. 8. Twenty-eight female B6D2F1 strain mice (6-8 wks) (approximately 20g each) were separated into four groups as follows: Group 1 contained nine mice injected with a 200 ul single bolus of 500 ng/ml human IFN-β (final dose of 100 ng/ mouse); Group 2 (nine mice) received 200 ul of an equivalent mass of PEG30kD-IFN-β; Group 3 received 200 ul of an equivalent mass of PEG(2 x 20 kD)-IFN-β; and Group 4 is a group of three uninjected mice serving as a negative control. Blood samples (approximately 200 ul/sample) were collected at nine indicated times by disruption of the retro-orbital venous plexus with a capillary tube. Blood samples were allowed to clot for 1 hr at room temperature, rimmed and microcentrifuged. Sera removed therefrom were stored at -70°C until all samples were collected. Sera were assayed for the presence of bioactive human IFN-β using the Toray assay. The results indicate that the area under the curve (AUC) is markedly enhanced in the PEG-IFN samples versus free IFN-beta and that PEG-IFN samples versus free IFN-β and that PEG[2 X 20 kD]-IFN-β is superior to the PEG[30 kD]-IFN-β.

Subcutaneous Administration

Mice were injected subcutaneously with IFN-β and PEG-IFN (100 ng/mouse). Figure 9 demonstrates that the total area under the curve (AUC) is dramatically enhanced for the PEG-IFN samples as compared with free IFN-β. The pharmacokinetic studies are consistent with the PEG-IFN samples having a longer half-life and increased AUC.

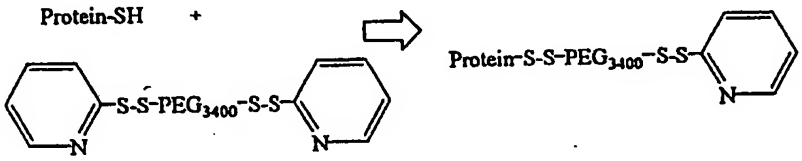
Example 5: Attachment of Low Molecular Weight PEG Moiety to Polypeptide

Tagging Interferon-beta with OPSS-PEG_{2k}-Hydrazide



Recombinant human interferon-β was provided in solution at 0.33 mg/ml in 50mM sodium acetate buffer, pH 3.8. Approximately 3.6 mg (40 mole excess to moles of protein) of the heterobifunctional PEG reagent, OPSS-PEG_{2k}-hydrazide, in 2 ml deionized water was added to 3 ml of IFN-β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 1 hour at 45°C. The reaction mixture was then analyzed with capillary electrophoresis to determine the extent of modification. Typical yields ranged from 90-97% that depended on the purity of the interferon β and PEG reagent. The solution was next loaded onto a size exclusion column (Superdex 75, Pharmacia) and eluted with 5 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE. The monoPEGylated interferon-β fractions were pooled together than used in a further modification step with high molecular weight PEG.

Tagging Interferon-β with $(OPSS)_2$-PEG_{3400}

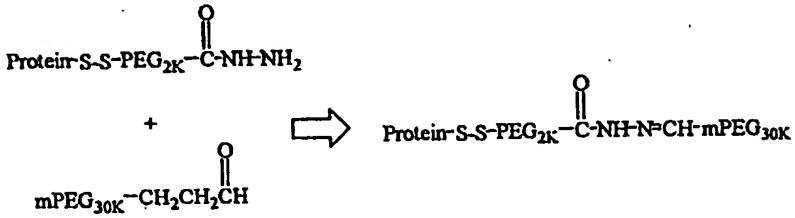


Recombinant human interferon-β was provided in solution at 0.33 mg/ml in 50 mM sodium acetate buffer, pH 3.8. Approximately 6.1 mg (40 mole excess to moles of protein) of the homobifunctional PEG reagent, $(OPSS)_2$-PEG_{3400}, in 2 ml deionized water was added to 3 ml of interferon-β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction was monitored with non-reducing SDS-PAGE and the final reaction mixture was analyzed with capillary electrophoresis to determine the extent of modification. Typical modifications for this reaction with interferon-β were >95%. The solution was then loaded onto a

size exclusion column (Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents. The monoPEGylated interferon-β fractions were combined.

EXAMPLE 6: Attachment of Second PEG Moiety to Low Molecular Weight PEGylating Polypeptide

Modification of IFN-S-S-PEG_{2k}-Hydrazide with $mPEG_{30k}$-Aldehyde (ALD)



To the combined fractions of IFN-S-S-PEG_{2k}-Hydrazide in Example 5 was added $mPEG_{30k}$-ALD in a 20 mole excess to protein. The reaction was conducted at room temperature (25°C) for 4 hours and a sample was added to a size exclusion column (Superose 6, Pharmacia) to determine modification yield. The modification yield of this reaction was typically >80% depending upon the purity of the PEG reagent and reaction conditions.

Having now fully described this invention, it will be appreciated that by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the

essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

WHAT IS CLAIMED IS:

1. A polyol-interferon-β conjugate having a polyol moiety covalently bound to Cys^{17} of human interferon-β.

2. The polyol-interferon-β conjugate according to claim 1, wherein said polyol moiety is a polyalkylene glycol moiety.

3. The polyol-interferon-β conjugate according to claim 2, wherein said polyalkylene glycol moiety is a polyethylene glycol (PEG) moiety.

4. The polyol-interferon-β conjugate according to any of claims 1-3, wherein the polyol-interferon-β conjugate has the same or higher interferon-β activity as native human interferon-β.

5. A process for producing the polyol-interferon-β conjugate of claim 1, comprising the steps of:

reacting interferon-β with a thiol-reactive polyol agent to site specifically and covalently attach a polyol moiety to Cys^{17} of human interferon-β to produce a polyol-interferon-β conjugate; and

recovering the produced polyol-interferon-β conjugate.

6. The process according to claim 5, wherein the thiol-reactive polyol agent is a thiol-reactive PEGylating agent.

7. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is mono-methoxylated.

8. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is bifunctional.

9. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is a polyol derivative having a functional group selected from the group consisting of orthopyridyl disulfide, vinyl sulfone, maleimide, and iodoacetimide.

10. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is an orthopyridyl disulfide derivative of a mono-methoxylated polyol.

11. The process according to claim 5, wherein the reacting step is carried out at an acidic pH where interferon-β is stable.

12. A pharmaceutical composition, comprising a polyol-interferon-β conjugate according to any one of claims 1-3, as an active ingredient, and a pharmaceutically acceptable carrier, excipient or auxiliary agent.

13. A method for treating infections, tumors and autoimmune and inflammatory diseases, comprising administering an effective amount of the pharmaceutical composition according to claim 12 to a subject in need thereof.

14. A method for stepwise attachment of polyethylene glycol (PEG) moieties in series to a polypeptide, comprising the steps of:

reacting a polypeptide with a low molecular weight heterobifunctional or homobifunctional PEG moiety having the following formula:

$$W\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}X,$$

where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide; and

reacting the low molecular weight PEG moiety attached to the polypeptide with a monofunctional or bifunctional PEG moiety to attach the monofunctional or bifunctional PEG moiety to a free terminus of the low molecular weight PEG moiety and form a PEG-polypeptide conjugate.

15. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has the following formula:

$$Y\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}Z,$$

wherein Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety attached to the polypeptide and Z is $-OCH_3$ or a group reactive with X to form a bifunctional conjugate.

16. The method according to claim 15, wherein the monofunctional or bifunctional PEG moiety is methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG.

17. The method according to claim 14, wherein W and X are independently selected from the group consisting of orthopyridyl disulfide, maleimides, vinylsulfones, iodoacetamides, hydrazides, aldehydes, succinimidyl esters, epoxides, amines, thiols, carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin.

18. The method according to claim 14, wherein the low molecular weight PEG moiety has a molecular weight in a range of about 100 to 5,000 daltons.

19. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has a molecular weight in a range of about 100 daltons to 200 kilodaltons.

20. The method according to claim 14, wherein the low molecular weight PEG moiety and/or the monofunctional or bifunctional PEG moiety is a copolymer of polyethylene glycol.

21. The method according to claim 20, wherein the copolymer of polyethylene glycol is selected from the group consisting of polyethylene glycol/polypropylene glycol copolymers and polyethylene glycol/poly(lactic/glycolic acid) copolymers.

22. The method according to claim 14, further comprising a step of purifying the PEG-polypeptide conjugate following the stepwise attachment of two PEG moieties in series to a polypeptide.

23. The method according to claim 22, wherein said step of purifying comprises one or more purification techniques selected from the group consisting of ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

24. The method according to any one of claim 14-23, wherein the polypeptide is interferon-β.

25. Use of the PEG-polypeptide conjugates produced by the method according to any one of claims 14-23 as a medicament.

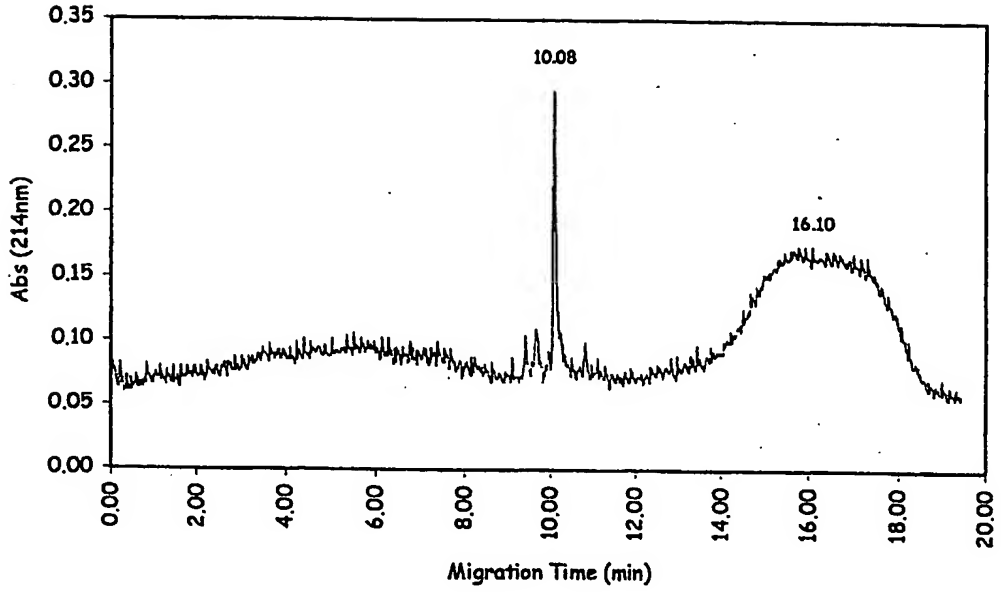

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Abs (214nm)
10.08
16.10
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Migration Time (min)

FIG. 1

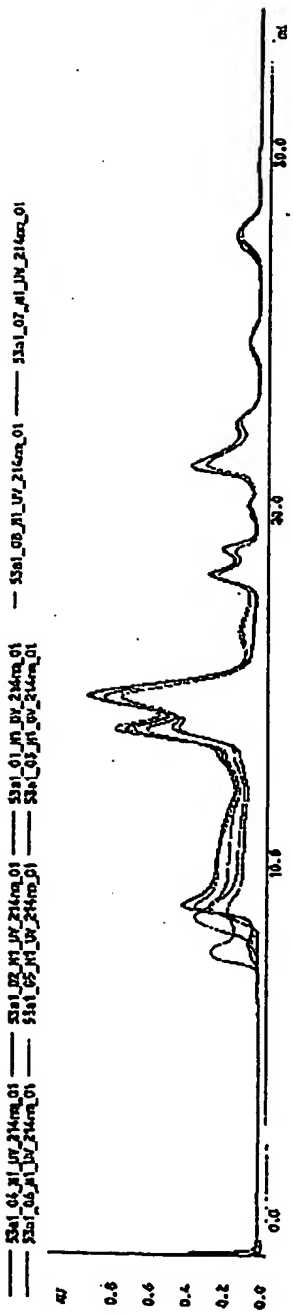


FIG. 2A

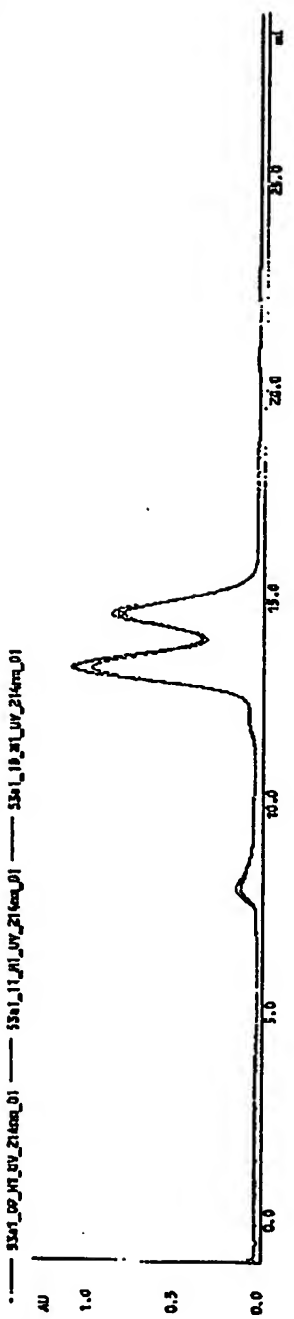


FIG. 2B

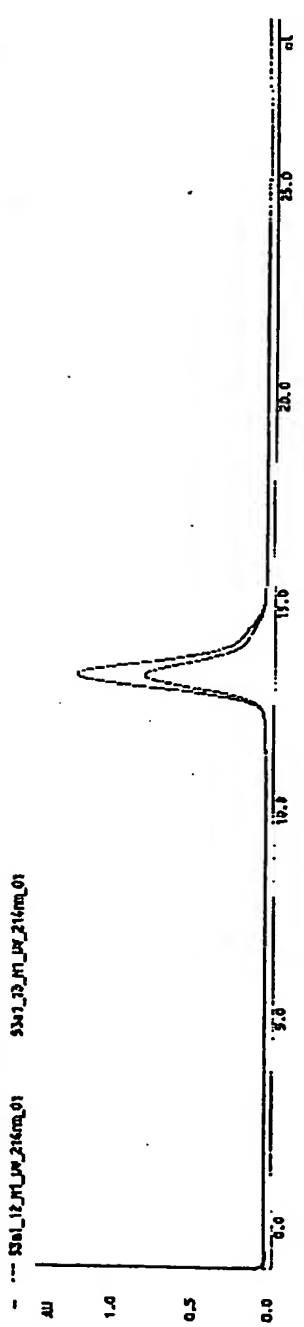


FIG. 2C

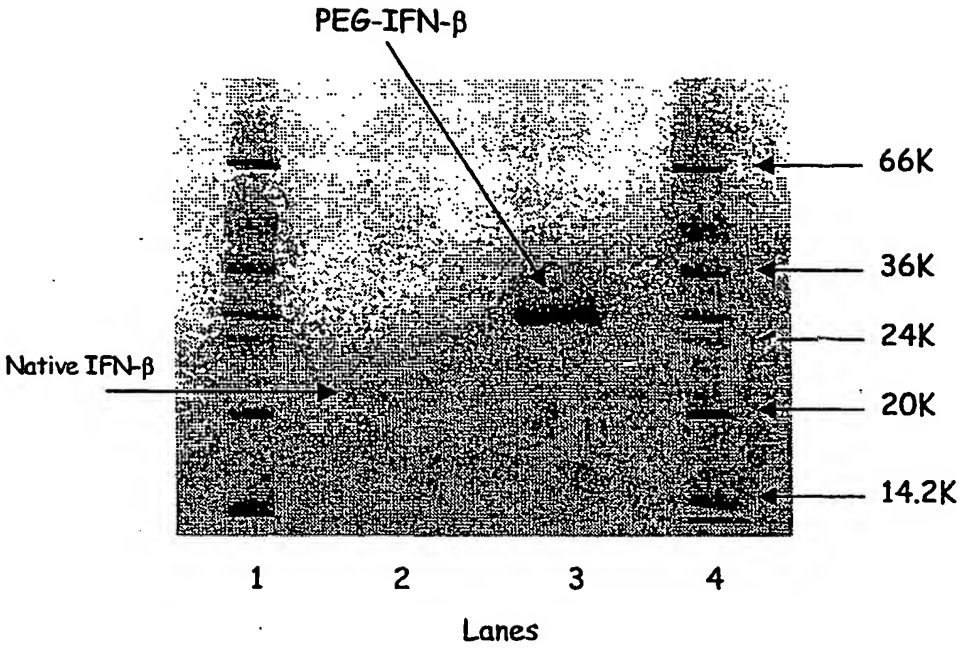

PEG-IFN-β
66K
36K
24K
Native IFN-β
20K
14.2K
1
2
3
4
Lanes

FIG. 3

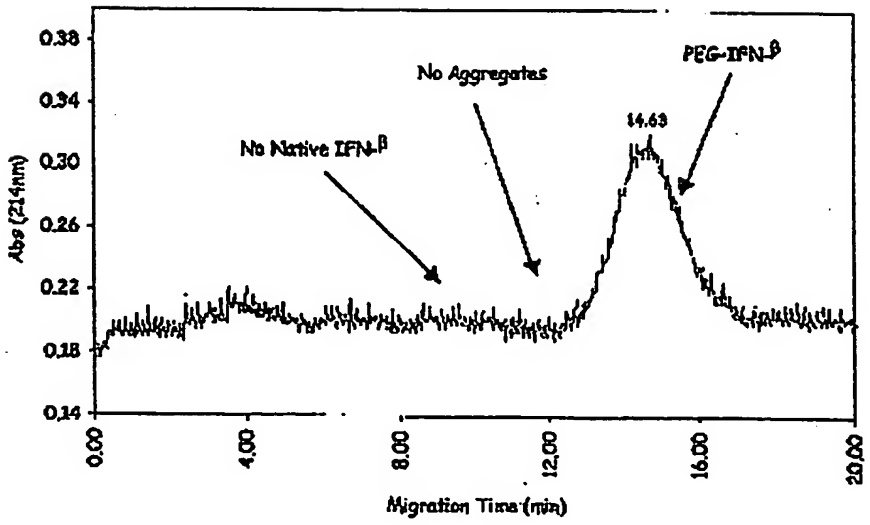

0.38
0.34
0.30
0.26
0.22
0.18
0.14
Abs (214nm)
No Native IFN-β
No Aggregates
14.63
PEG-IFN-β
0.00
4.00
8.00
12.00
16.00
20.00
Migration Time (min)

FIG. 4

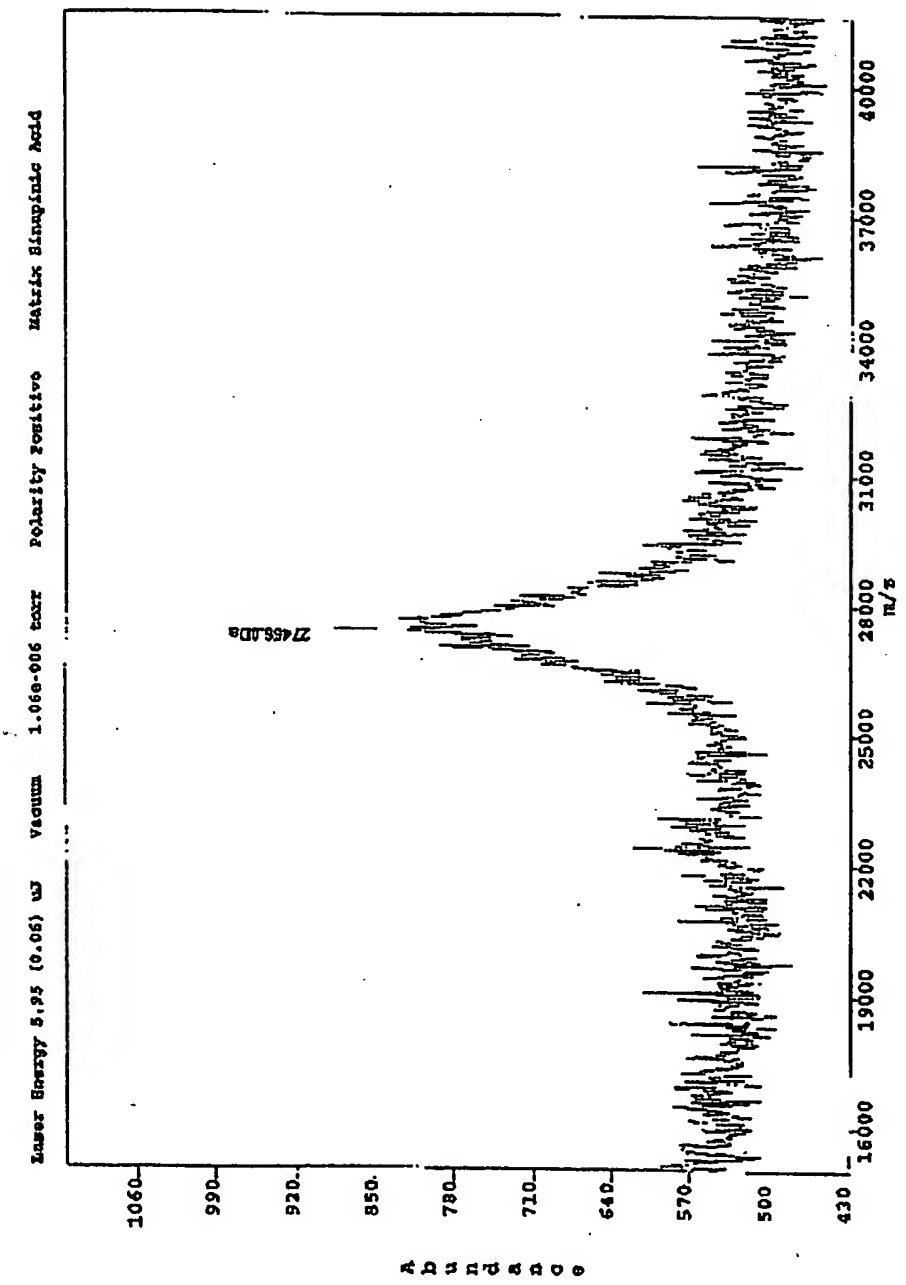

Laser Energy 5.95 (0.06) u7 Vacuum 1.06e-006 torr Polarity Positive Matrix Sinapinic Acid
27456.0Da
Abundance
m/z
1060
990
920
850
780
710
640
570
500
430
16000
19000
22000
25000
28000
31000
34000
37000
40000

FIG. 5

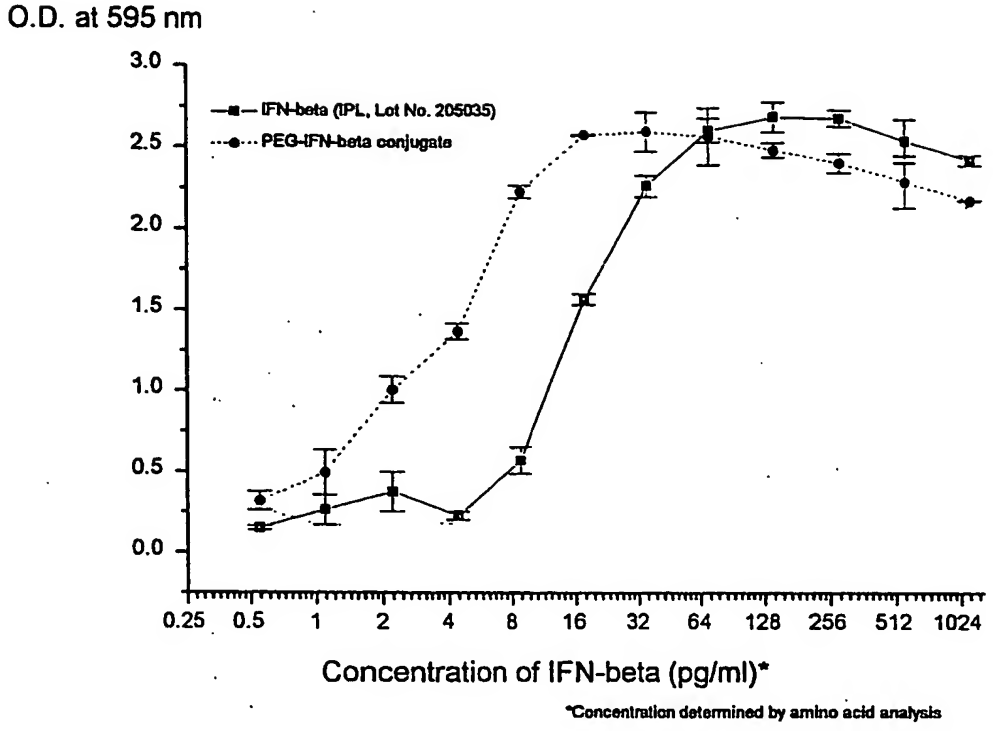

O.D. at 595 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IFN-beta (IPL, Lot No. 205035)
PEG-IFN-beta conjugate
0.25 0.5 1 2 4 8 16 32 64 128 256 512 1024
Concentration of IFN-beta (pg/ml)*
*Concentration determined by amino acid analysis

FIG. 6

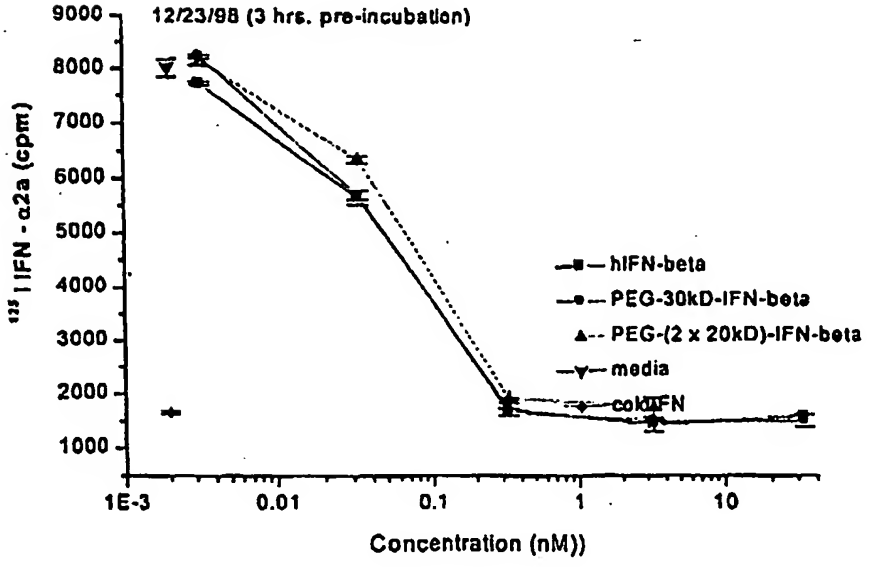

12/23/98 (3 hrs. pre-incubation)
125 I IFN - α2a (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
1E-3 0.01 0.1 1 10
Concentration (nM))
hIFN-beta
PEG-30kD-IFN-beta
PEG-(2 x 20kD)-IFN-beta
media
cold IFN

FIG. 7

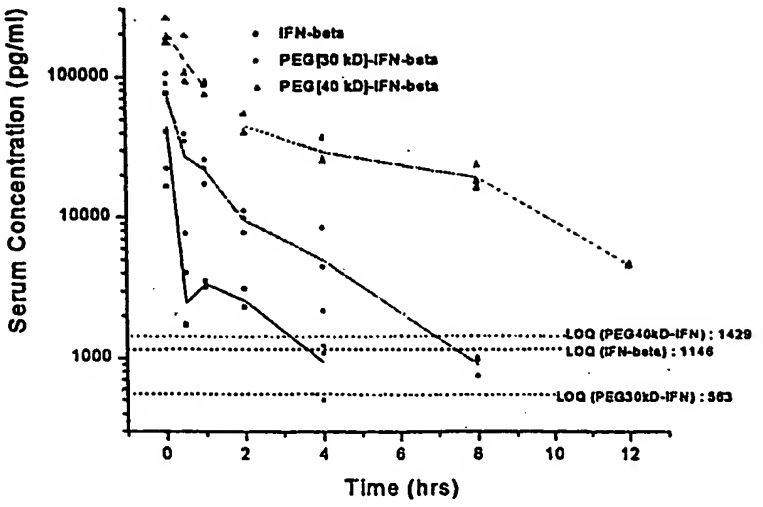

Serum Concentration (pg/ml)
100000
10000
1000
IFN-beta
PEG[30 kD]-IFN-beta
PEG[40 kD]-IFN-beta
LOQ (PEG40kD-IFN) : 1429
LOQ (IFN-beta) : 1146
LOQ (PEG30kD-IFN) : 563
0
2
4
6
8
10
12
Time (hrs)

FIG. 8

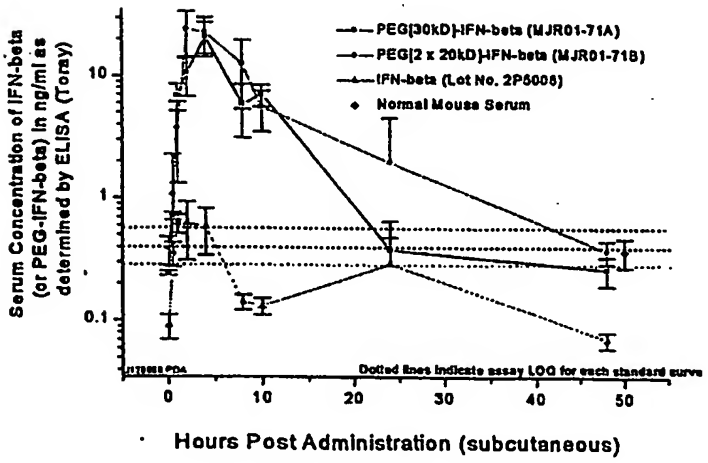

Serum Concentration of IFN-beta (or PEG-IFN-beta) in ng/ml as determined by ELISA (Toray)
10
1
0.1
PEG[30kD]-IFN-beta (MJR01-71A)
PEG[2 x 20kD]-IFN-beta (MJR01-71B)
IFN-beta (Lot No. 2P5006)
Normal Mouse Serum
Dotted lines indicate assay LOQ for each standard curve
0
10
20
30
40
50
Hours Post Administration (subcutaneous)

FIG. 9

Organization TC 1600 Bldg/Room REMSEN

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